Diagnostic Use of Hair Analysis for the Detection of Misuse of Amfetamines and Cannabinoids

Thesis submitted in Accordance with the Requirments of the University of Glasgow for the Degree of Doctor of Philosophy

by

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Dedication

TO MY PARENTS

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FAMILY

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Analytical Methods

IA	Immunoassay
TLC	Thin-layer Chromatography
FID	Flame Ionisation Detection
NPD	Nitrogen-Phosphorous Detection
ECD	Electron Capture Detection or Electro Chemical Detection
HPLC	High-Performance Liquid Chromatography
UV	Ultraviolet
DAD	Diode Array Detection
LC	Liquid Chromatographic
EI	Electron-Impact
SIM	Selected Ion Monitoring
SPE	Solid-Phase Extraction
LLE	Liquid-liquid Extraction
EMIT	Enzyme Multiplied Immunoassay Technique
RIA	Radio Immunoassay
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
FPIA	Fluorescence Polarization Immunoassay
CE	Capillary Electrophoresis
LC–MS	Liquid Chromatography Mass Spectrometry
LC–MS-MS	Liquid Chromatography–Tandem Mass Spectrometry
GC –MS	Gas Chromatography Mass Spectrometry
GC-MSD	GC-Mass Selective Detection
GC-FID	GC with Flame-Ionisation Detection
GC-MS-EI	GC-MS Operating in Electron Impact Mode
GC -MS-MS	GC –Tandem Mass Spectrometry
GC-MS-NCI	GC-MS in Negative Chemical Ionisation
GC-MS/MS-NCI	GC-Tandem Mass Spectrometry in Negative Chemical Ionisation
GC-MS/MS-PCI	GC-Tandem Mass Spectrometry in Positive Chemical Ionisation
SFE	Supercritical Fluid Extraction
PCI	Positive Chemical Ionisation
TIC	Total Ion Current

SIM	Selected Ion Monitoring
LOQ	Limit of Quantitation
LOD	Limit of Detection
STA	Systematic Toxicological Analysis
CV	Coefficient of Variation
SD	Standard Deviation
mS	milli Sieverts
abv	Absolute Volume

Drugs and materials

AF	Amfetamine
MA	Methamfetamine
MDA	3, 4 Methylenedioxyamfetamine
MDMA	3, 4 Methylenedioxymethamfetamine
MDEA	3, 4 Methylenedioxyethylamfetamine
6-MAM	6-Monoacetylmorphine
PCP	phencyclidine
MBDB	N-methylbenzodioxazolylbutanamine
PMA	Para methoxyamfetamine
PMMA	Para-methoxymethamfhetamine
4-MTA	4-Methylthioamfetamine
MeOH	Methanol
EtOH	Ethanol
SDS	Sodium dodecylsulfate
TFA	Tritluoroacetic acid anhydride
CBD	Cannabidol
CBN	Cannabinol
Δ^9- THC	Δ^9 –tetrahydrocannabinol
Δ^9 –THCA	Δ^9 –tetrahydrocannabinolic acid
Δ^8- THC	Δ^8 –tetrahydrocannabinol
Δ^9 –THCCOOH	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol
11-OH-THC	11-Hydroxy-Δ^9-tetrahydrocannabinol
CB1and CB2	Cannabinoids Receptors
NaOH	Sodium Hydroxide
KH_2PO_4	Potassium Dihydrogen Phosphate
HCl	Hydrochloric Acid

EtOAc	Ethyl Acetate
DI H_2O	Distilled Water
Cl^-	Chloride ion
Na^+	Sodium ion
Mg^{2+}	Magesium ion
Ca^{2+}	Calcium ion
SO_4^{2-}	Sulphate ion
K^+	Potassium ion
PFPA	Pentafluoro propionic anhydride
PFP	Pentafluoroproionyl
PFPOH	Pentafluoropropanol
PSA	Propionic acid anhydride
TFA	Trifluoroacetic anhydride
CDFA	Chlorodifluoroacetic anhydride
HFB	Heptafluoro-n-butyryl
MBTFA	*N*-methyl-*bis*-trifluoroacetamide
TMS	Trimethylsilyl
TMSIm	Trimethylsilylimidazole
TBDMS	Tert-Butyldimethylsilyl
EDTA	Ethylenediaminetetraacetic acid
D.C.M	Dichloromethane
PH	Phenyl

Other

SOFT	Society of Forensic Toxicologists
SOHT	Society of Hair Testing
Rpm	Revolutions per minute
R^2	Linear Correlation Coefficient
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
SAMHSA	Substance Abuse and Mental Health Services Administration
ADHD	attention deficient hyperactivity disorder
CNS	Central Nervous System
t_R	Retention time
m/z	Mass/Charge Ratio
BCS	British Crime Survey

PAR	Peak Area Ratio
Cmax	Maximum Blood Concentration
Tmax	Time to Maximum Concentration
d	dextrorotatory
l	levorotatory
S or R	The configuration of the groups bonded to the chiral atoms

Summary

The Security Forces Hospital, Saudi Arabia analyses approximately 10,000 urine samples per year. One of the main problems with urine sample collection is the possibility of adulteration. It has been found that 2-3 % of samples have had attempts at adulteration, usually by adding water. In cases of suspected adulteration, the possibility of analysing a hair sample in conjunction with the corresponding urine sample would be beneficial to confirm the validity of the specimen results. From available statistical records in Security Forces Hospital, Saudi Arabia [1], the most common drugs of abuse used were amfetamines and cannabinoids. These represent about 80-90% of total positive results of drugs of abuse. The purpose of this study was to develop a single method to analyse these drugs in hair and to apply this to case samples received from The Security Forces Hospital, Saudi Arabia.

First of all, mass fragments and retention times for amfetamines and cannabinoids were identified by GC-MS using derivatisation agents, PFPA / ethyl acetate (2: 1 v/v) and PFPA / PFPOH (1: 0.75 v/v) for amfetamines and cannabinoids, respectively. The results showed good peak shape, good chromatographic resolution and good sensitivity for amfetamine (AF), methamfetamine (MA), 3, 4-Methylenedioxyamfetamine (MDA), 3, 4-Methylenedioxymethamfetamine (MDMA), 3, 4- Methylenedioxyethylamfetamine (MDEA), Δ^9 –tetrahydrocannabinol (Δ^9 –THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Δ^9 -THC-COOH) compounds.

The comparison of the efficiency of four different pre-treatment methods (enzymatic, alkaline, acid and methanol) to extract AF, MA, MDA, MDMA, MDEA, Δ^9 –THC and Δ^9 -THC-COOH from hair samples obtained from known amfetamines and cannabinoids abusers was investigated. Each method was followed by SPE, derivatisation and detection using gas-chromatography mass-spectrometry. The method giving the best recovery for the detection and quantification of cannabinoids and amfetamines simultaneously was to be selected and fully validated.

The preliminary results demonstrated difficulty with the cannabinoids recovery and the lower concentrations of standards were not detected using any of the pre-treatment methods. Consequently, the calibration curves for all pre-treatment methods could not be plotted and the initial method was deemed insufficiently sensitive for cannabinoid detection purposes. In order to obtain a better recovery of the cannabinoids, parameters in

the method were modified. Four different eluents were compared for the cannabinoids; acetone /chloroform (1:1 v/v), hexane / EtOAc (9:1 v/v), hexane / EtOAc (8:2 v/v) and methanol using alkaline pre-treatment and the pH of the phosphate buffer was also changed (pH5, 6, 7 and 8).

However, the problem still persisted with the cannabinoids recovery. Meanwhile, the amfetamines concentration showed no significant change throughout this investigation.

As a result of the poor cannabinoids recovery, only the amfetamines were investigated. For the comparison study, only one hair sample positive for amfetamine was available so the pre-treatment comparison study was based on the recovery of AF using the four pre-treatment methods.

The positive hair sample was separated into portions and pre-treatment methods, alkaline (1M NaOH), β-Glucuronidase (*helix pomatia*), methanol (MeOH) and acid (0.1M HCl) were used on these and compared. The best recovery for amfetamine was obtained using the β-glucuronidase pre-treatment method and this extract was also found to be cleaner than the alkaline and methanol pre-treatments. β-glucuronidase pre-treatment was selected as the method of choice for the extraction of amfetamine content in hair. The method was validated to include linearity, recovery, intra- and inter-day precision, limit of quantitation (LOQ) and limit of detection (LOD) for all five amfetamine compounds. The method was shown to be reliable and robust for these substances.

The stability of AF in hair was investigated to assess the validity of analysing hair samples for the presence of AF in victims of drowning. Ten amfetamine positive hair samples were submerged in fresh and sea water for different periods of time. The drug concentrations in the samples were monitored over a period of 8 weeks. Hair samples were analysed using the validated method. The results showed a significant decrease of amfetamine in hair with the time submerged in sea water. Fresh water had a much less significant effect over the study period.

The validated method was successfully applied to 16 case samples obtained from living volunteers with a known history of fenethylline (AF precursor) abuse and 6 post-mortem case samples where amfetamines had been detected in the post-mortem blood.

1 Introduction

1.1 Overview of drug use

The problem of drug use and abuse has existed for a very long time. Over the last 200 years, most of the countries in the world have suffered from this problem. Differences vary from country to country but the epidemic has risen with time. There is no doubt that there are serious negative effects of drug abuse on the health and security of the social structure of all nations. It estimated that 30,000 premature deaths per year in the United States are attributed to illegal drugs and the numbers are growing continually [2]. The official statistics of the British Crime Survey (BCS) in 2004/5 indicated that 34.5% (approximately 11 million people) of 16 to 59 year olds in England and Wales had used at least one illicit drug in their lifetime. It was reported that the extent of cannabis use decreased between 2003/04 and 2004/05, and amfetamines use decreased between 1998 and 2004/05 in England and Wales among this age group (16 to 59 year old) [3].

1.1.1 Drug of Abuse

There are various definitions of a "drug of abuse" Most of them focus mainly on the use of a substance for some purpose other than that intended. For example a drug which is used therapeutically but can cause harmful effects when taken irresponsibly. Illicit drugs are restricted by the law, with possession or supply carrying legal penalties. As such they are prohibited by governments [4].

1.1.2 The Misuse of Drugs Act 1971

The Misuse of Drugs Act 1971 is an Act in the United Kingdom and its aim is to control the possession and supply of numerous listed drugs and drug-like substances whether they be illegal drugs or prescription medications and to enable international co-operation against illegal drug trafficking. There are different penalties for possession and supply of controlled drugs under the Misuse of Drugs Act depending on the nature of the offence. These are summarised in Table 1.1. This Act allows regulation in the use of some controlled drugs by various professional people in different fields such as doctors [5].

Table 1.1 Penalties under the Misuse of Drugs Act [6]

Class	Drugs	Penalties
A	Ecstasy, LSD, Heroin, Cocaine, Crack, Magic Mushrooms, Amfetamines (if prepared for injection)	**Possession**: Maximum of 7 years in prison and/or unlimited fine **Supply**: Up to life in prison and/or unlimited fine
B	Amfetamines, Methylphenidate (Ritalin), Pholcodine	**Possession**: Maximum of 5 years in prison and/or unlimited fine **Supply**: Maximum of 14 years in prison and/or unlimited fine
C	Cannabis, Tranquillisers, some Painkillers, Gamma hydroxybutyrate (GHB), Ketamine	**Possession**: Maximum of 2 years in prison and/or unlimited fine **Supply**: Maximum of 14 years and/or unlimited fine

1.1.3 Control Drugs Classification

In the United Kingdom, illegal drugs are classified into three classes A, B and C depending on their potential for abuse toxicity. These regulations are continually amended whether adding new drugs, upgrading or downgrading these drugs in each class [5]. Class A has the most harmful drugs and highest penalties and Class C drugs are the least harmful and have the lowest penalties. The regulations of controlled drugs for possession, supply, production and importation or exportation were made under this Act to meet the medical or scientific needs. These are categorised as follows:

Schedule 1: Drugs which are the most stringently controlled, not for medical use and usually for research purposes for example cannabis, coca leaf, ecstasy, LSD and raw opium.

Schedules 2 and 3: Drugs which are illegal to possess without prescription. Schedule 2 drugs include amfetamines, cocaine, dihydrocodeine, diconal, heroin, methadone,

morphine, opium in medicinal form, pethidine and ritalin. Schedule 3 includes barbiturates, rohypnol and temazepam tranquillisers.

Schedule 4: Part 1. Drugs which are illegal to possess without a prescription, for example, most minor tranquillisers. Part 2. Drugs which are legal to possess in their medicinal form without a prescription, but it is illegal to supply them to other people, for example, anabolic steroids.

Schedule 5: Drugs which are allowed to be sold over-the-counter at a pharmacy without a prescription, and may be bought by anyone with impunity, for example, cough medicines, anti-diarrhoea agents and mild painkillers [7].

1.2 Forensic Toxicology

1.2.1 Introduction

Toxicology is the comprehensive study of poisons and drugs, concerned with their effects on living organisms and dealing with the diagnosis of poisoned people.

Forensic toxicology is the study of the effects of drugs and poisons on human beings in the context of the law. The use of toxicological investigations for the purpose of medico-legal enquiries for living and deceased people depends on the country [8, 9].

A toxicologist deals with different biological samples such as urine, blood, hair, sweat, saliva, other body fluids and organs such as liver, kidney and brain, to investigate the presence of a toxic substance[9]. Urine is the most common specimen used for drug testing [10].

The main objective of toxicological analysis is to prove or exclude the presence of undetermined toxic substances in biological matrices and accurately identify them. The analyst must be capable of identifying and quantifying these substances within a specified time period [11].

1.2.2 Systematic Toxicological Analysis (STA)

Systematic Toxicological Analysis (STA), is a process which involves the identification and quantitation of unknown substances [11]. To analyse drugs in conventional biological matrices, Systematic Toxicological Analysis involves three stages, pre-treatment and extraction, detection and identification.

1.2.2.1 Pre-treatment and Extraction

The biological samples undergo pre-treatment in STA and this pre-treatment depends on the type of the sample, for instance whether they are biological fluids or tissue samples. Buffer solutions are widely used for dilution of plasma and serum [12]. Hydrolysis and protein removal are used in the preparation of biological samples. Pre-treatment is often carried out by enzyme preparations like β-glucuronidase or strong acid and basic agents which are necessary to split conjugated metabolites. Enzyme pre-treatment is preferable because strong acid and base can cause substances to decompose. Protein removal can also cause low recoveries due to drug molecules being bound to the precipitated protein.

An extraction method is required to concentrate drugs of interest and to remove any matrix compounds. This is done using a variety of techniques such as Liquid-Liquid Extraction (LLE) and Solid-Phase Extraction (SPE). In order to achieve optimum results extraction procedures should have high recoveries, be capable of separately extracting a wide variety of target analytes (acidic, neutral and basic), have clean extracts and be reproducible and rapid. SPE can be used for the extraction of single drugs or groups of related drugs, and has become the most popular extraction technique [11].

1.2.2.2 Derivatisation Methods

Analysis of drugs by GC or GC–MS may require derivatisation to make substances appropriate for analysis. It is essential for the analysis of polar and thermally labile compounds to make them volatile and thermally stable. The main purpose of the derivatisation step is to improve chromatographic properties such as better peak shapes and increased resolution from related compounds. This is achieved by increasing the volatility and stability, while decreasing the polarity of the compounds. Another benefit is often the formation of ions at higher mass/charge ratios, giving more diagnostic mass spectra. Greater sensitivity can be obtained by introducing groups of high electron affinity (such as halogen atoms), these increase the ionisation efficiency. The optimum derivatisation

reaction should be simple, rapid and complete, and produce a stable, single derivative with good reproducibility. The derivatisation of different functional groups on analytes can include hydroxyl groups, ketones, carboxylic acids and amines. The three most common derivatisation methods are silylation, acylation and alkylation.

1.2.2.2.1 Silylation

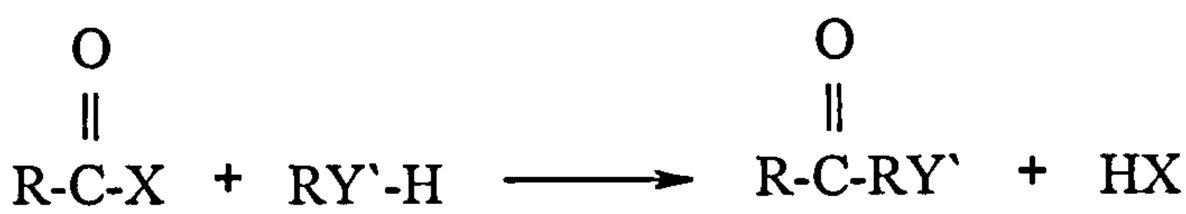
Silylation occurs when an active proton in –OH, –SH or –NH groups is displaced with an alkylsilyl group from the silylation reagent. Some protic functional groups on the compound are converted to either silyl ethers or esters. The ability of various functional groups to form silyl derivatives was found to be as follows, alcohols > phenols > carboxylic acids > amines > amides. Trimethylsilylation is the most commonly used procedure for silylation. There are a variety of trimethylsilylating reagents available such as trimethylhalosilanes, TMS-amines, TMS-esters and TMS-amides, which are varied in their properties, for instance: volatility, reactivity and selectivity. MSTFA is the most volatile TMS-amide used to form TMS derivatives of alcohols and phenols. Trimethylsilylimidazole (TMSIm) is another silylation reagent used for hydroxyl and carboxyl groups and tert-Butyldimethylsiyl (TBDMS) derivatives are used to increase hydrolytic stability and to give useful mass spectrometric fragmentation. All silylation reagents and derivatives are sensitive to moisture [13]. This is the general silylation reaction in the formation of trialkylsilyl derivatives [14]:

$$R_3Si\text{-}X + R`\text{-}H \rightarrow R_3Si\text{-}R` + HX$$

1.2.2.2.2 Acylation

Acylation occurs by introducing an acyl group on to a molecule containing a reactive hydrogen. There are many functional groups that may be acylated to produce derivatives such as alcohols, amines, amides, thiols, phenols, enols, sulfonamides. The acylation reactions can be carried out by acyl halides, acid anhydrides or reactive acyl derivatives. acyl halides are highly reactive. Perfluoroacyl derivatives such as trifluoroacetyl (TFA), pentafluoroproionyl (PFP) and heptafluorobutyryl (HFB) are the most commonly used. These perfluoroacyl derivatives lead to an increase in the abundance of high m/z ions [13].

The general acylation reaction is as follows [14]:



1.2.2.2.3 Alkylation

Alkylation is another commonly used derivatisation method, which replaces an active hydrogen as in carboxylic acids, alcohols, thiols and amines with an alkyl or an aryl group. Alkyl halides, iodides (methyl, ethyl, propyl, isopropyl) or benzyl and most substituted benzyl bromides are the most common reagents used to obtain alkyl derivatives [13]. One type of alkylation reaction is as follows [14]:

1.2.2.3 Detection

After biological sample preparation, the analysis of drugs in biological matrices usually involves several analytical techniques for qualitative or quantitative analysis of drugs. There are several types of commercial techniques available. For screening purposes immunoassays (IA) and occasionally thin-layer chromatography (TLC) can be used. For confirmation purposes gas chromatography (GC), with a detector such as flame ionisation detection (FID), nitrogen-phosphorous detection (NPD), electron capture detection (ECD) and mass spectrometry (MS) can be used. Also high-performance liquid chromatography (HPLC), with detectors such as ultraviolet spectrometry (UV), diode array detection (DAD), electrochemical detection (ECD) and mass spectrometry (MS) can be used for confirmation [15].

GC-MS is the technique of choice in forensic toxicology and can be used for STA [11]. The major advantages are good sensitivity and specificity.

1.2.2.4 Identification

Commonly encountered drugs have well defined mass spectra which are reproducible between instruments. Identification of unknown substances is not easy due to the large number of possibilities. Comprehensive libraries do exist but need to be updated regularly to cover as many drugs as possible.

1.2.3 Solid Phase Extraction

1.2.3.1 Introduction

SPE is a physical extraction process for rapid sample preparation in which a solid (stationary phase) is used to selectively extract, concentrate, and purify the analyte of interest in the biological matrix prior to chromatographic analysis.

SPE was introduced commercially in 1970 as a technique for urine sample preparation and in 1979 the small size columns of modern SPE was first used. Its applications included pharmaceutical, industrial, environmental, clinical, and forensic fields for the extraction of substances. In the last few years, there has been a trend toward replacing the classical LLE by SPE due to its potential advantages, there are visible practical advantages of SPE compared to LLE which could be useful in drug extraction, including the following:

- high specificity
- high analyte recoveries
- cleaner extracts and enhanced removal of interferences and particulates, lead to less equipment contamination
- elimination of emulsions
- better reproducibility of results
- reduced solvent consumption, as well as reduction in hazardous and environmental wastes
- ease of use and the SPE extraction can be automated
- enhanced analytical sensitivity and reduced LOD and LOQ
- lower sample quantities and shorter sample extraction times [12, 16, 17]

As a result of the difficulty in extracting a wide range of drugs by a single mode column, the mixed mode bonded silica SPE columns such as Bond Elut Certify™ and CleanScreen™ can be used. These columns contain both hydrophobic and cation

exchange functional groups properties, which allow the extraction of a broad range of drugs including acidic, neutral and basic drugs by selectively eluting with various elution solvents. The majority of reviews have focused on the use of SPE to extract individual drugs or classes of drugs [12].

1.2.3.2 Principles of Solid Phase Extraction

SPE sorbent (base support) plays a major role in SPE extraction efficiency and separation quality, there are various sorbent materials in use like silica or bonded silica, alumina and polymers, but bonded silica is the most popular sorbent used [12, 16].

The functional group bonded to the silica determines the interaction mechanism (extraction) between the functional groups of the sorbent with the analyte of interest. The common interaction mechanisms between sorbent and analytes are as follows:

1.2.3.2.1 Non-polar Interactions (Reversed phase)

Non-polar interactions are carried out between the hydrocarbon chains of hydrophobic or polar organic analytes and the sorbent. These interactions are caused by low energy Van der Waals (dispersion forces). Saturated hydrocarbon chains such as C_{18} and C_8, or aromatic rings such as phenyl (PH) are usually used in non polar sorbents. These sorbents have the ability to retain a wide range of organic compounds, due to the non specificity of the interaction [12, 16].

1.2.3.2.2 Polar Interactions (Normal phase)

Polar interactions are used to extract polar analytes from non-polar organic solvents. The analytes are retained on the sorbent due to hydrogen bonding, dipole-dipole and π-π interactions between polar analytes and the polar sorbent. A high degree of specificity is obtained by optimizing the polarity of the conditioning solvent and the elution solvent [12, 16].

1.2.3.2.3 Ion Exchange Interactions

Ion exchange interactions take place between charged sorbents and the analytes from low ionic strength aqueous or organic samples with the opposite charges, for example a negatively charged sorbent (cation exchangers) will retain positive charged analytes and in

contrast a positively charged sorbent (anion exchangers) will retain negatively charged analytes. This mechanism has a high degree of specificity, therefore, the contamination in the matrix is easy to wash out [12, 16].

1.2.3.3 Solid Phase Extraction steps

1.2.3.3.1 Column Preconditioning

To prepare the column sorbent to receive and interact with analytes, the column sorbent needs suitable conditioning prior to the sample application in order to improve reproducibility and recovery. This is done using polar solvents to activate and open up the coiled hydrophobic portion of the sorbent. The sorbent conditioning can vary depending on the type of analyte [12, 18].

1.2.3.3.2 Sample Application

Sample application follows the column precondition. A low flow rate is required to allow the drugs of interest to have adequate time to interact with the sorbent, consequently more drugs are retained on the column and selectively eluted, thereby increasing recovery [12].

1.2.3.3.3 Column Wash

In order to remove endogenous components rather than the drugs of interest which have bound to the sorbent during sample application, the column is washed. An appropriate wash solvent has to be chosen very carefully in order to avoid loss of the target analyte during the wash process. At this step, the pH of the column is adjusted as some of the drugs are dependant on the pH for their elution [12].

1.2.3.3.4 Column Drying

Residual water should be removed from the SPE column prior to the elution of the drugs of interest and prior to analysis by washing with a suitable organic solvent or drying the sorbent with air under vacuum. The presence of water prevents the optimal elution of the drugs as a result of the immiscibility of the elution solvent with water, on the other hand, the presence of water may react with the stationary phase of the column, consequently causing damage to the GC column [12, 17].

1.2.3.3.5 Elution of Relevant Drugs

The final step in extraction of the drugs of interest should be elution with the appropriate solvents. These have to be strong enough to elute the drugs of interest completely with a small eluent volume and should be selective to avoid elution of interfering compounds [12].

2 Hair Analysis

2.1 Introduction

Hair analysis is useful for detecting and monitoring past drug use over a period of weeks to several months depending on the length of the hair sample and on the stability of the drug in the hair specimen. It has been shown to be useful in the detection of chronic drug abuse in forensic and clinical applications and has been used for the purpose of employment screening, monitoring compliance for drug treatment programs, in cases involving criminal responsibility, detection of drug use in utero and the passive exposure of the fetus, in cases of driving license suspension and in the investigation of abstinence for chronic drug abusers. Hair analysis can be used as a complementary technique to urine and blood analysis which show relatively recent usage. The combined information from these types of sample and hair will provide information regarding acute and chronic use [19].

The exact time of drug ingestion cannot be determined from hair analysis results. The estimation of the ingestion time is only possible in intervals of months not in weeks, days or hours. This is because there are many varying factors involved in the incorporation of drugs into hair [20]. These are discussed later on in section 2.5. The time of drug consumption can be approximated in months by knowing the rate of hair growth in an individual and the location of the drug along the hair shaft [21].

The first poisonous substance to be determined in hair was arsenic in 1857 by Casper. It was found in the hair of a body exhumed eleven years after burial [22]. In 1954, the first organic drug (AF) was detected in guinea-pig hair [23]. Due to the capability of atomic absorption spectroscopy to detect low concentrations of elements (nanograms per milligram), hair analysis was used in 1960s and 1970s to detect toxic heavy metals, such as lead or mercury in hair [24].

The first report of the detection of opiates (heroin/morphine) in hair was reported in 1979 using a radioimmunological method. This publication was the first method which dealt with drugs of abuse in human hair and it led to many further investigations in this area [22]. The first paper to confirm radioimmunological results with a chromatographic method was printed in 1980 [25]. The availability of more sensitive and selective analytical methods allowed for the detection of low levels of drugs which were incorporated into the hair in the picogram per milligram range.

The role of hair drug testing in toxicological applications has been widely discussed by different scientific communities such as the Society of Forensic Toxicologists (SOFT) and the Society of Hair Testing (SOHT) [26]. These scientific conferences are important to address controversial issues surrounding hair analysis. SOHT in 2004 set out recommendations for testing of drugs in hair. A consensus was reached to include points on sampling, shipping and storage, decontamination, hair disintegration, the screening test, criteria for mass spectrometric analysis, specific drug classes (opiates, cocaine, amfetamines, cannabinoids), internal quality control, external quality control [27]. These provide guidelines for laboratories carrying out hair analysis for drugs.

The potential advantages of hair analysis over other conventional or unconventional biological specimens are:

- collection of hair specimens is easy.
- sampling is less intrusive. It is less embarrassing than urine collection which requires close supervision.
- adulteration is difficult.
- a second sample can be obtained if there is any doubt about the integrity of the sample.
- long time stability of hair specimens at room temperature. This means they can be stored and transported indefinitely without refrigeration and quick analysis is not required.
- it has a wider window of detection than blood or urine. The window of detection will depend on the length of the hair.
- periods of abstention can be identified.
- parent drugs and metabolites are incorporated into hair. Urine and blood samples often only have the metabolites present [28-31].

2.2 The Anatomy and Physiology of Hair

2.2.1 Introduction

The interpretation of drug concentration results in hair is complex. To avoid the misinterpretation of results, an understanding of hair biology such as the anatomy, physiology, biochemistry and growth cycle is necessary to provide the correct scientific interpretation of the drug results in the hair.

2.2.2 Structure of hair

Hair is a cylindrical shaft which grows from a small sac called the hair follicle. Hair varies morphologically from individual to individual, within the same individual as well as within a single region within an individual. Hair emerges from the skin either in single strands or in groups [32]. Hair consists of three layers of cells as shown in Figure 2.1.

The cuticle is the outermost layer of the hair shaft, which plays a role in anchoring the hair shaft in the hair follicle and acts to protect the interior layers of the hair shaft. The cuticle consists of a single layer of elongated, overlapping cells and is free of pigment. The thickness and length of each cuticle cell is around 0.5 to 1.0 μm and 45 μm, respectively. Exposure of the hair to harmful substances and environments such as chemical, heat, light or mechanical treatment can damage or destroy the cuticle. The cuticle acts as a strong barrier against the loss of drug integrity. When the cuticle is damaged drugs may be removed more easily from the hair [19, 33, 34].

The cortex is the middle layer and the bulk of the hair shaft. It contains lengths of keratinised cells and also melanin granules which are the principle pigment in hair, eyes and skin. Hair colour depends on the quantity, distribution and type of pigment in hair. In the cortex there are small air spaces between the cortex cells called fusi which are filled with fluid and replaced by air as the hair grows and dries out [33].

The medulla is the innermost layer of the hair shaft. This layer exists in human hair and can be continuous, discontinuous or absent. The medulla layer contains many keratinized cells. Intra and intercellular air spaces are also found in this layer as well [32, 33]. Melanin granules may also be present in the medulla.

There are two types of melanin in human hair, ears and in certain brain structures in man. They play an essential role in the protection of internal tissues against UV radiation [35]. Their structures can be very simple or very complex [36] and some of these are still not fully understood [37]. In hair they are attached to the protein matrix [38].

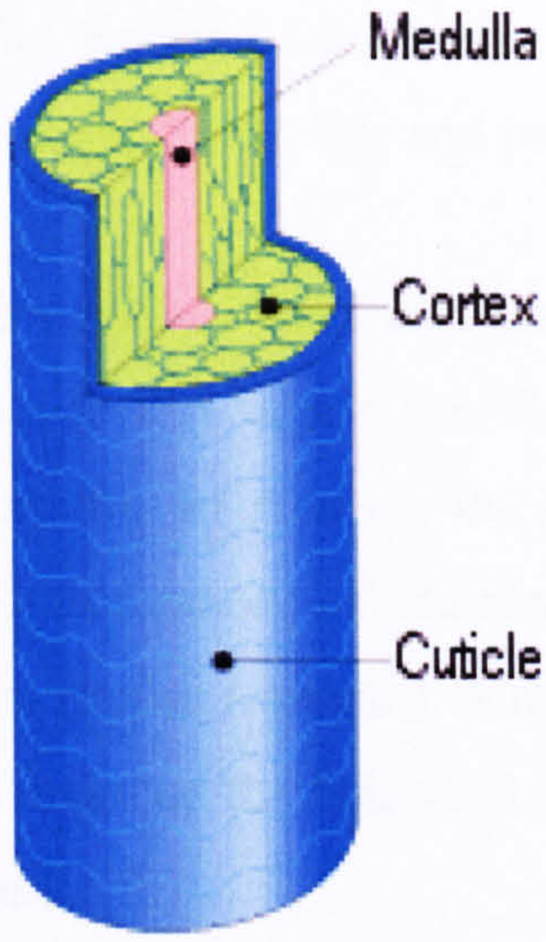


Figure 2.1 Cross-section of a hair shaft [39]

2.2.3 Hair Composition

Human hair contains proteins called keratin that are bound together tightly to form long fibres, producing a stable structure. The proteins are rich in sulphur. Proteins, water, lipids and minerals represent approximately 65 to 95%, 15 to 35%, 1 to 9% and 0.25 to 0.95% of hair content respectively. These percentages depend on the moisture in the hair. Trace elements and heavy metals are also present.

2.2.4 The Hair Follicle

The hair follicle is a small sac that surrounds the hair root. It is found 3 to 4 mm below the skin's surface and is embedded in the epidermal epithelium of the skin.

Sebaceous and apocrine glands surround the hair follicle. Their ducts secrete into the follicle. In addition to these glands, the eccrine sweat gland also surrounds the hair follicle in axillary and pubic areas. Its duct does not secrete into the follicle but near to the exit of the hair follicles, onto the surface of the skin. The secretion of these glands may be a source of trace elements and drugs in hair. The sebaceous gland and eccrine sweat gland

are located and distributed near the surface of the body. The apocrine gland is located in the axilla, the external auditory meatus, the eyelids and perineal region.

There are three regions in the hair follicle. The first region is the innermost region (lower segment) in and around the bulb. In this region the biological synthesis of hair cells is carried out. The second region (middle segment) is the keratogenous region. This region extends from above the bulb. Keratinization, hair hardening and solidification take place in this region and the final region (upper segment) is the permanent hair region. In this region dehydrated cornified cells make up the hair shaft. Figure 2.2 shows a cross section of the skin surrounding a hair follicle [33].

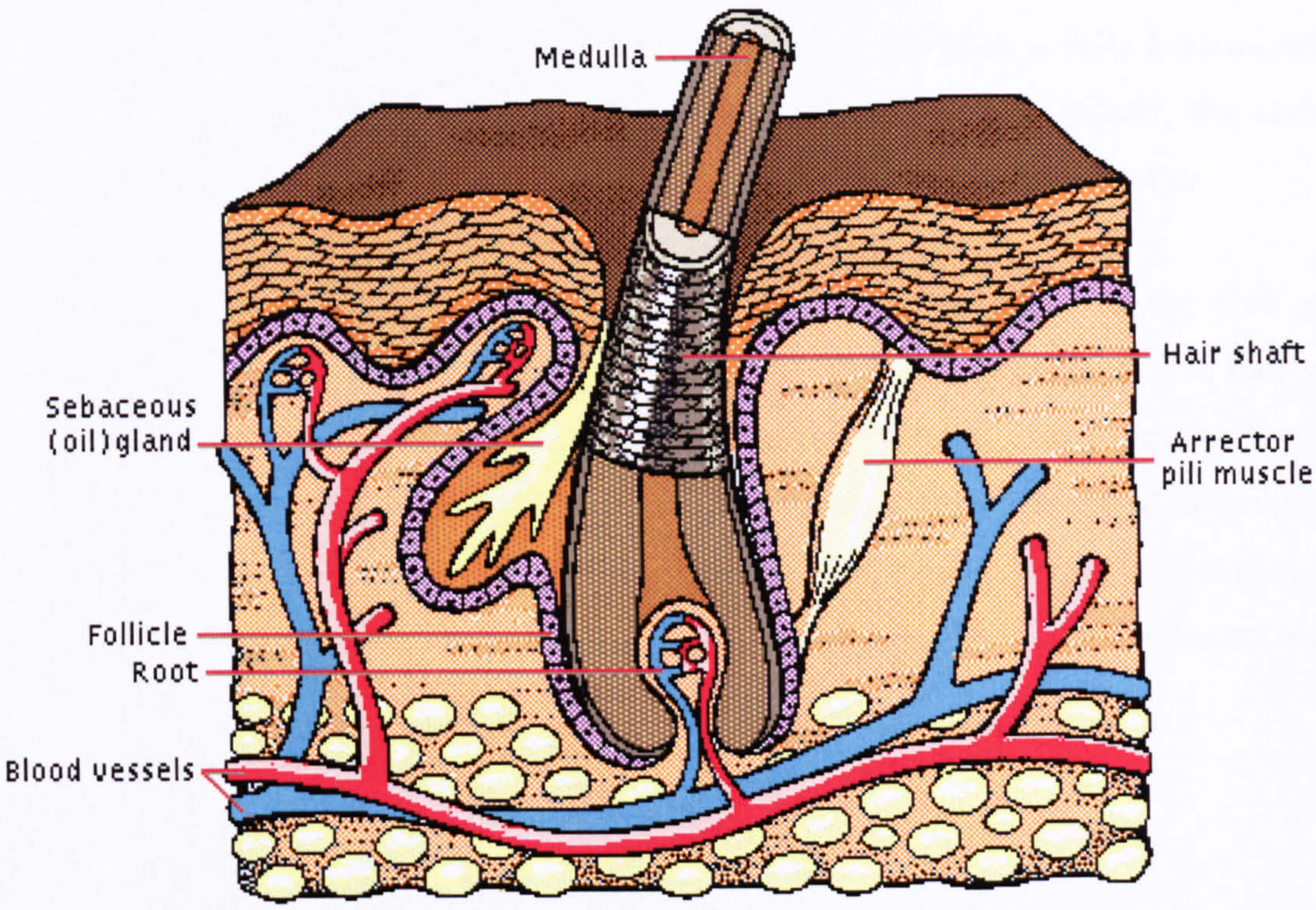


Figure 2.2 A cross section of the skin surrounding a hair follicle [40]

2.2.5 The Hair Growth Cycle

Hair grows in a cycle which consists of three phases:

The first phase is the anagen phase. It is known as the growth phase due to the metabolic activity of the matrix cells increasing and hair being produced in the follicle. Hair cells are produced and the keratinzation process begins. This phase lasts around 2 to 3 years and its growth rate is dependent on the anatomical region. The nutrients and extraneous materials such as trace metals and drugs supplied by capillary blood are incorporated into the hair shaft at this phase.

The second phase is the catagen phase. It is a transition period of about 4 to 6 weeks, between active growth and a resting phase. This phase produces a fully keratinized root which is club end formed and separate from the bulb of the hair follicle, the club hair becomes a white node, then cell division stops and the follicle becomes shorter.

The third phase is the telogen phase. This phase is characterized as the club and is completely separated from the bulb becoming close to the scalp surface. No hair grows and it is easily removed by pulling. It lasts a period of between 2 to 3 months depending on the hair type and age. The anagen phase and telogen phase represent approximately 85% and 10 to 15% of hair growth on the scalp of adult respectively, in the catagen phase some hair is passed. A new hair shaft begins to grow, while the old hair is forced out [19, 33, 41]. Figure 2.3 shows the hair growth cycle phases.

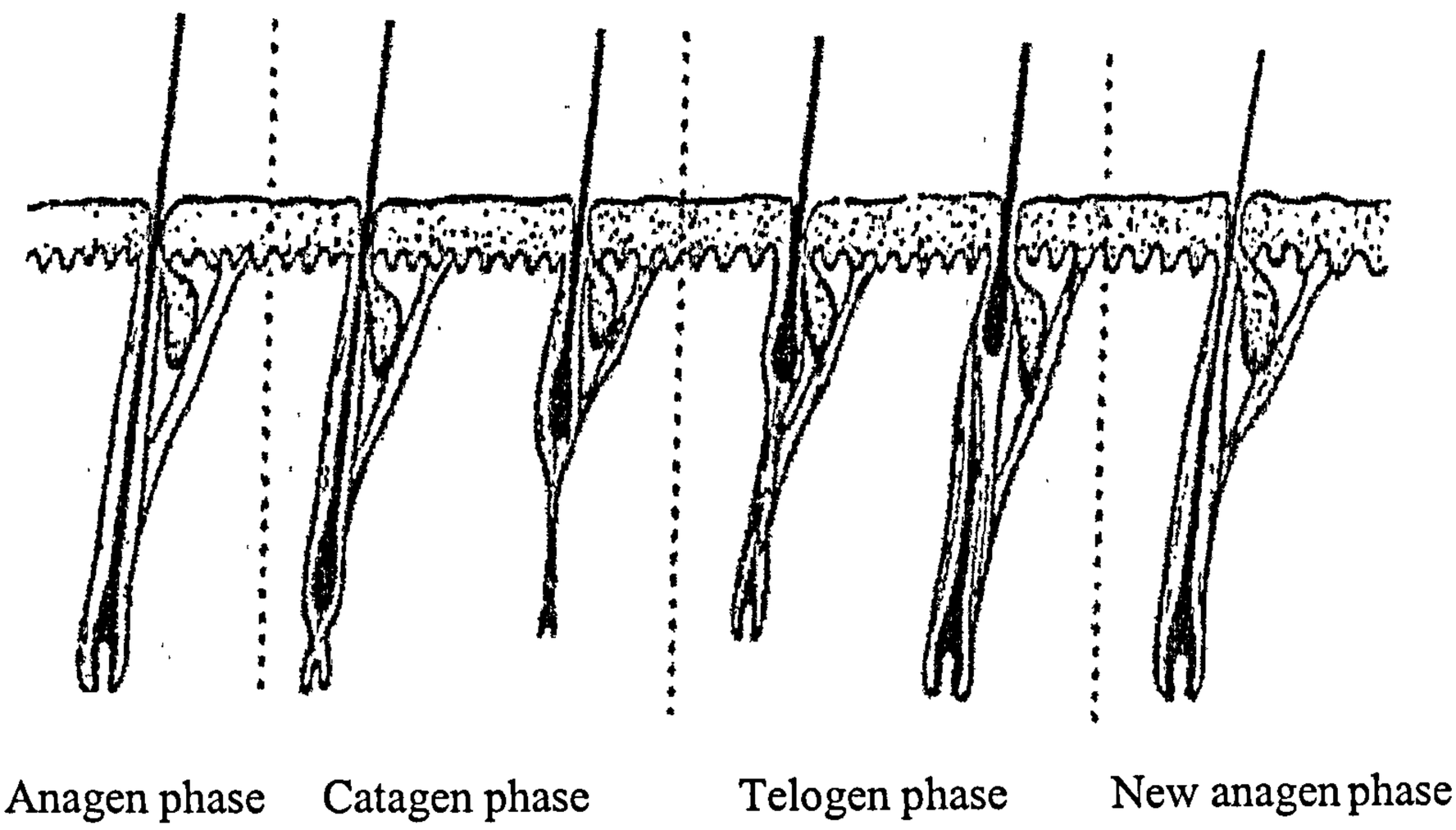


Figure 2.3 Hair growth cycle phases [34]

2.2.6 Rate of hair growth

Hair growth rate varies between individuals and also within an individual. Thus, the precise determination of hair growth rate is difficult. The average growth rate of human head hair is 1 cm/month. In general, hair growth rate depends on a number of factors such as hair type and anatomical location. For example, scalp hair grows much faster than hair in the pubic or axillary regions which in turn grows faster than beard hair. Many others factors affect the hair growth rate. These include race, sex and age. Scalp hair in women tends to grow faster than in men and there is a decrease of hair growth rate as adults age. Therefore, these factors have to be taken into account when interpreting hair drug results [33]. The time taken for the drug to appear in the hair shaft at the skin's surface for a medium growth rate of 1 cm/month was estimated to be between 9 and 14 days. It was reported in another study that codeine was detected between 15 and 21 days after a 120 mg dose for a segment >1 cm from the skin surface [34].

2.2.7 Types of Hair

There are three basic hair types on the human body. These are vellus, terminal and intermediate and each has their own individual properties. These differ in length, texture, colour, diameter and shape. Vellus hair is fine, short, non-pigmented and has a small cross-sectional area. This type of hair is found on the eyelids, the forehead and on the bald scalp. Terminal hair is coarse, long, pigmented hair with a large cross-sectional area found in scalp, beard, eyebrow, eyelash, armpit and pubic areas. Intermediate hair is of intermediate length and shaft size and is found on the arms and legs of adults.

There are three kinds of follicles which produce different hair types on the human body. Intermediate hair is produced from nonsexual hair follicles which are not influenced by hormones and do not change after puberty. While ambosexual hair follicles are influenced by hormones and therefore will change during puberty. These affect hair in the axilla, pubic regions and on the temple area of the scalp. Male hair follicles exist in males and affect hair in beard area, ears, nose, chest, abdomen and top of the head [33].

2.3 Hair sample collection

The posterior vertex region, at the back of the head, is the preferred site of collection of a hair sample rather than other samples such as beard, axillary, pubic hair and other areas of the head. This is because this region is characterized with a high percentage of follicles in

the anagen phase, which are a uniform distance from the scalp. This is especially important if sectional analysis is required. The highest and the fastest growth rate is also found at the vertex region in the scalp, up to 3.4 cm/month. Beard hair has the slowest growth rate (approximately 0.8 cm/month) and pubic hair is also slower than scalp hair (0.9 cm/month). In this area the effect of age and sex on the incorporation of drugs have less influence. Pubic hair and axilliary hair is used as an alternative to scalp hair when it is not available. However the problem of possible exposure to contamination by urine and sweat should be recognised [19, 33, 34, 41].

The different concentrations of drugs in the pubic, axillary and scalp regions has been reported in various studies and these differences are attributed to a number factors such as presence of apocrine glands, blood supply, different telogen/anagen ratios and hair growth rate [24].

2.4 Drug incorporation into hair

In order to interpret hair analysis results accurately, the incorporation of drugs into hair has to be considered. At present, the exact understanding of the incorporation of drugs into hair is unknown. The transfer of drugs from the body to hair is suggested and carried out by various proposed mechanisms at different times of hair growth cycle.

The first mechanism is simple passive diffusion, in which drugs transfer from the blood into the growing hair cells and bind tightly with the interior of hair shaft during keratogenesis (during formation).

The second mechanism suggested is transfer from sweat, sebaceous and apocrine gland secretions (after formation).

The third proposed method is from the external environment such as smoke, powder and absorption by skin after formation and when the hair has emerged from the skin. Incorporation of drugs into hair from tissues surrounding the hair follicle and deep compartments in skin which can accumulate lipophilic drugs in the epidermis and the hypodermis layers cannot be excluded. Figure 2.4 shows the different proposed mechanisms of drug incorporation during different phases [42, 43].

It was reported that codeine was detected in the hair root 30 minutes after administration and detected in distal scalp hair for up to 10 weeks after a single dose of the drug. The study was only carried out for a period of 10 weeks [21].

It has been reported that methamfetamine (MA), methylendioxymethamfetamine (MDMA) and phencyclidine were found in rat hair root within 5 minutes of administration. Thus, the hair root can be used as an indicator of recent drug use. It has been reported that the concentration of drug in hair is dependent on the amount of drug incorporated into hair and retained in hair, where some of the incorporated drug is redistributed outside the hair shaft [44].

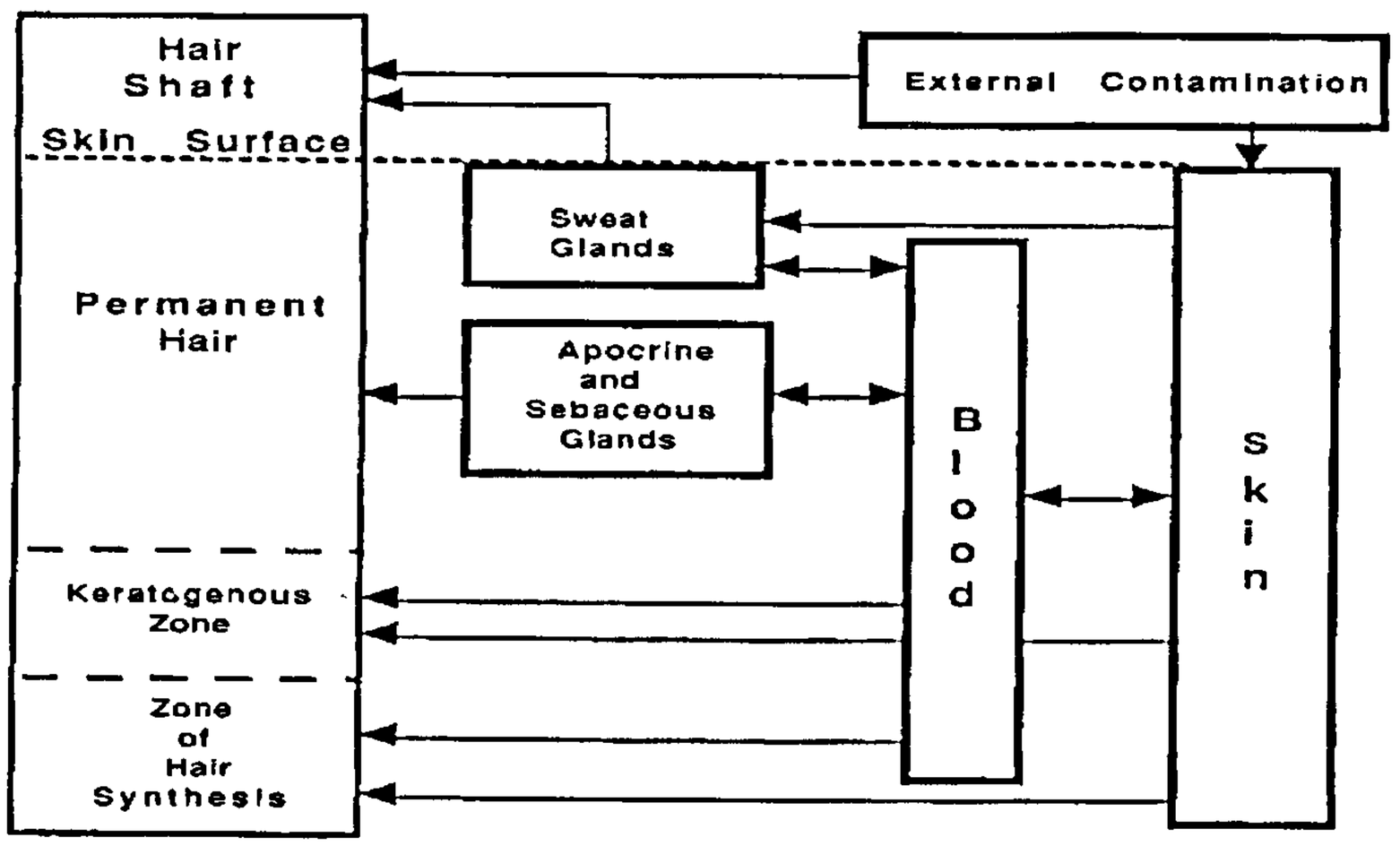


Figure 2.4 Diagram showing the possible mechanisms for drugs incorporation into the hair [42]

2.5 Factors affecting drug incorporation into hair

Lipophilicity, melanin affinity and pKa are important factors that may influence the incorporation of drugs into hair. For example, basic drugs are readily incorporated into hair, while acidic drugs exhibit a weak incorporation into hair [45]. Another factor which has been suggested to influence the incorporation of drugs into hair is colour. For example, the drug concentrations tend to be higher in hair that contains pigment than hair

that does not. It has been reported that cocaine was incorporated in the hair of African American females with black hair at concentrations higher than in the hair of Caucasian males or females with coloured hair. This has been reported as racial bias. Other possible factors are gender differences, the structure of the hair such as fine hair versus coarse hair [21, 46, 47] and cosmetic treatment (colouring , bleaching , perming) [48].

In addition, pK_a plays a major effect in drug transport and incorporation of drugs into hair. For example the difference in pH between plasma (7.4) and the matrices cells like melanocytes (3-5) leads to the accumulation of basic drugs in the matrices cells, due to a negative concentration gradient of penetrable non protonated basic drugs from plasma to the cytosol of the matrices cells. In contrast, acidic drugs like Δ^9- tetrahydrocannbinol carboxylic acid will be transported in the opposite direction. This may be part of the reason why its concentration is low in hair matrices. The binding of drug with melanin is affected by pH as well [34].

2.6 Hair Decontamination

The contamination of hair from external sources is a problem in hair analysis for the detection of drugs [43, 49-52]. Decontamination procedures are used to remove non-drug contaminants such as dirt and oils from hair but are also necessary to eliminate external contamination prior to hair analysis to avoid false hair positive results from passive contamination for example from being in the environment of drug smoke or from handling drugs and then hair.

There is no set standard procedure to remove contamination from hair. Currently, different solutions such as water, Tween, SDS, other detergents, phosphate buffer, methanol, ethanol, acetone, dichloromethane, isopropanol are used in various combinations to decontaminate the hair. However, it is difficult to fully validate decontamination procedures. This is because it is not clear if all of the contaminant is being completely removed or if drug which has been incorporated through use is also being removed. It has been reported that the decontamination of hair depends on the hair type, where certain types are more resistant than others in removing environmental contamination. For example thick black hair was demonstrated to be more resistant to decontamination than other hair types. The basic concept of decontamination procedures is to remove potential drugs which are loosely bound to the surface. However, it has been shown that drugs from

passive contamination is not only on the surface of the hair but also present in the interior of the hair [50].

One study showed that decontamination procedures could not remove all external contamination of opiates (heroin, 6-MAM, morphine, acetylcodeine and codeine) by using 5 ml of dichloromethane for 5 min at room temperature three times. 5 mg of a powdered mixture of heroin hydrochloride and acetylcodeine (10:1 w/w) was applied to subjects' hands for 5 minutes and this was wiped onto the hair. This method of contamination may not be realistic. The authors attempted to use cut-off values and metabolite-to-drug ratios to evaluate sample contamination but concluded these methods were not practical. They suggest that the reporting of a positive hair result alone is not acceptable without other evidence for example further toxicology tests such as urinalysis or clinical data [43].

In contrast, another paper [53] reported an extensive decontamination procedure to remove external contamination from hair. Various experimental models such as soaking and coating with cocaine, morphine and 6-MAM, PCP and methamfetamine drugs were used. Attempts to identify the contamination of the hair sample were carried out by subtracting the multiplied value of the last washed fraction from the amount of drug found in the hair sample following digestion. When the results were less than the cutoff for the parent drugs the contamination was reported to be from external contamination and the result reported as negative. If the result was higher than the cutoff for the parent drugs this was reported to indicate active drug use and the result reported as positive.

The effect was evaluated for four different wash procedures on the quantitative measurement of cocaine and its metabolites from rat hair. The cocaine was administered by intraperitoneal injection. All four methods showed different drug concentrations in hair compared with unwashed hair. This showed that all four washes have the potential to remove systemic drug exposure from hair. This study proved that wash procedures are capable of removing environmental contamination and incorporated drug whether from active or passive use [54].

3 Amfetamines and Cannabinoids

3.1 Amfetamines

3.1.1 Introduction

β-phenethylamine derivatives such as amfetamine (AF), methamfetamine (MA) and 'designer drugs' such as methylenedioxy derivatives of amfetamine including 3, 4-Methylenedioxyamfetamine (MDA), 3, 4-Methylenedioxymethamfetamine (MDMA), 3, 4-Methylenedioxyethyl-amfetamine (MDEA), N-methylbenzodioxazolbutanamine (MBDB) and its methoxy derivatives for instance para-methoxyamfetamine (PMA), para-methoxymethamfetamine (PMMA) and 4-methylthioamfetamine (4-MTA) are powerful stimulants of the central nervous system [55]. AF and related compounds are listed in Classes A and B of the Misuse of Drugs Act 1971 [3].

The drugs are widely abused, primarily by young people, in different areas of the world. It is reported that AF and MDMA are the most common drugs used from this class of compounds and are used either alone or in combination with other illicit drugs [56]. Consequently, these drugs are of interest to forensic and clinical toxicologists and to sports scientists. Traditionally, these drugs would be detected in blood or urine specimens. However, with concerns relating to the human rights of individuals, privacy and dignity, less conventional biological specimens such as oral fluid, hair, sweat and nails are becoming more frequently used for drug screening, particularly in the workplace. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) the abuse of AF and related compounds comes second to cannabis misuse in Europe [57].

3.1.1.1 Amfetamine

AF is a central nervous system stimulant. It is available as amfetamine phosphate and amfetamine sulphate and is ingested as a racemic mixture [58]. It was first synthesised in 1887 and used during World War II to prevent fatigue and maintain alertness. It has been manufactured commercially since 1932. Its misuse was reported to have spread to Japan, Europe, Canada and the US in the 1960s. It can be abused by oral, nasal or intravenous routes [55, 59]. The clinical use of AF was limited to the treatment of narcolepsy in adults and attention deficient disorder with hyperactivity (ADHD) in children. Also it has been

used in decongestant preparations and as an appetite suppressant [60]. The popularity of AF competes with cocaine on the street as a result of it being cheaper and giving a similar effect. For example, 5-10 milligrams of dextroamfetamine is sufficient to ward off fatigue and produce mental and physical effects such as euphoria. Consequently, it is a popular illicit stimulant among students, truck drivers, night workers and athletes. Side effects of the drug include dryness of the mouth, sweating and hypertension [61].

3.1.1.2 Methamfetamine

MA is a strong central nervous system stimulant and is available as methamfetamine hydrochloride [58]. MA was synthesized in the early 1900s for clinical use. It was highly abused and caused problems in different countries such as the US, Mexico, South America, the Middle East, Asia and Australia. It is abused through smoking, insufflations, injection and oral ingestion. Clinically, its use is limited to weight loss programs and for the treatment of narcolepsy in adults and to treat attention deficit disorder in children and exogenous obesity [62-64]. It was reported that 4% of the US population has tried this drug during 2000 [65].

3.1.1.3 Methylenedioxy Derivatives

MDA, MDMA and MDEA are methylenedioxy derivatives of amfetamines. The derivatives were synthesised to circumvent the then legal controls on amfetamines. They are abused for similar reasons and have similar pharmacological actions to the amfetamines (see section 3.1.1.1). Also, they were found to have some hallucinogenic properties. Although there were claims that MDMA could be used diagnostically by psychiatrists, the drugs have no significant therapeutic use. They are available as methylenedioxyamfetamine hydrochloride, methylenedioxymethamfetamine hydrochloride and methylenedioxyethylamfetamine [58, 66]. Methylenedioxy derivatives of amfetamine or methamfetamine are abused to enhance understanding and empathy [67]. They became the most popular recreational drugs in the mid-1980s [55]. Within recent years, they have become the second most popular abused drugs in Europe and North America especially at dance clubs as "rave" drugs [68].

MDMA and MDA were first synthesised in 1914 and 1910, patented in 1914 and 1940 respectively. Since 1986, clandestine laboratories started to synthesise derivatives of MDMA and MDA [69]. Methylenedioxy derivatives of amfetamines are mostly consumed orally and originally were synthesised for medical use [70, 71]. Their popularity was due

to producing effects such as euphoria, enhanced energy, a desire to socialize and their reputation of being safe. Other studies, however, indicated some harmful effects to humans such as hepatotoxicity and neurotoxicity [72]. Following the use of the methylenedioxy derivatives of amfetamine the abuser may experience drowsiness, muscle aches, general fatigue, depression lasting 1–2 days and may have difficulty in concentrating [68].

It has been reported that the development of new designer drugs has increased during the last few years. Approximately 200 different derivatives have been described. A few of them are known to be used in Europe where the methylenedioxy derivatives of amfetamine and methamfetamine are the largest designer drugs group abused [73].

3.1.2 Physico - Chemical Properties of Amfetamines

Chemically, these compounds are derivatives of β-phenyl-isopropylamine. AF contains a primary amine and MA has a substituted amine. Methylenedioxy derivatives are ring-substituted. The basic structure of amfetamines and the structure of all five amfetamines used in this study are shown in Figure 3.1 and Figure 3.2. Most of them have pKa values of approximately 10. All are suitable for drug testing in non-conventional and conventional matrices. AF and related compounds have similar pharmacological and toxic effects such as increased alertness, insomnia, energy and decreased fatigue and appetite with enhanced mood and euphoria. The variations of AF and related compounds' structures have an effect on the active drugs properties, for instance psychomotor stimulant effects, an increased metabolic half-life and diminished hallucinogenic activity [55].

For medico-legal cases, particularly in relation to workplace drug testing, the effects of metabolically altering urinary pH and dilution of urine samples either physically or using a diuretic agent, must be taken in to account and this requires careful interpretation of the analytical results [74]. The properties of investigated amfetamines are shown in Table 3.1.

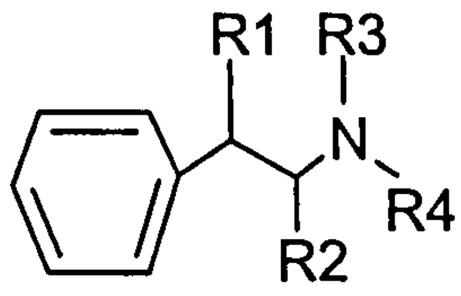
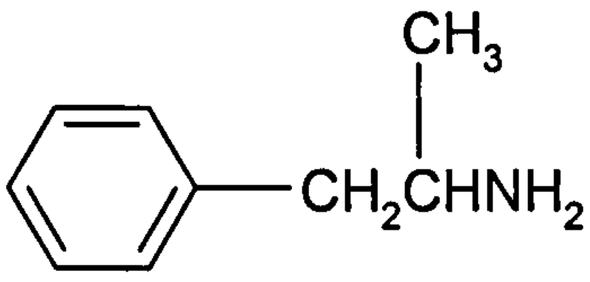
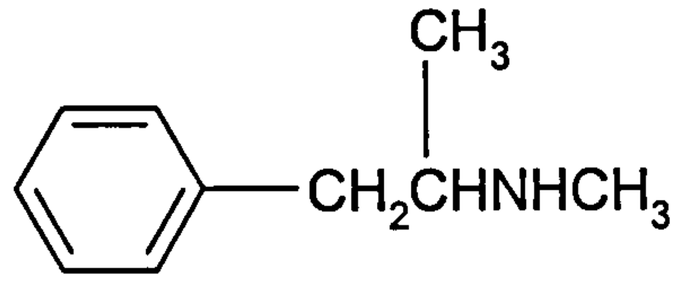


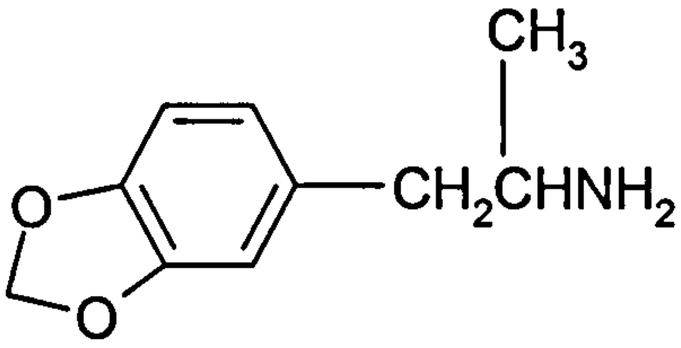
Figure 3.1 Amfetamine Basic Structure (β-phenyl-isopropylamine)



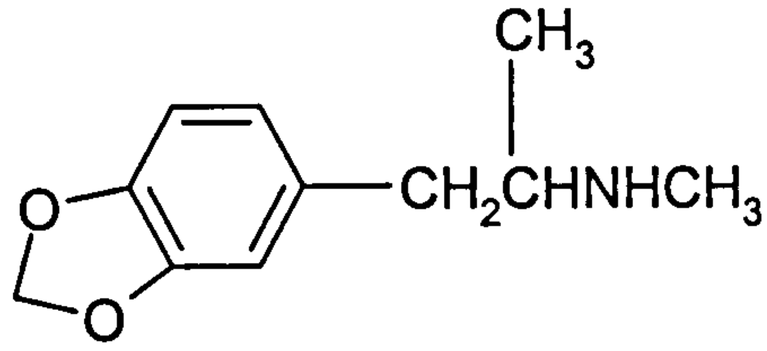
AF



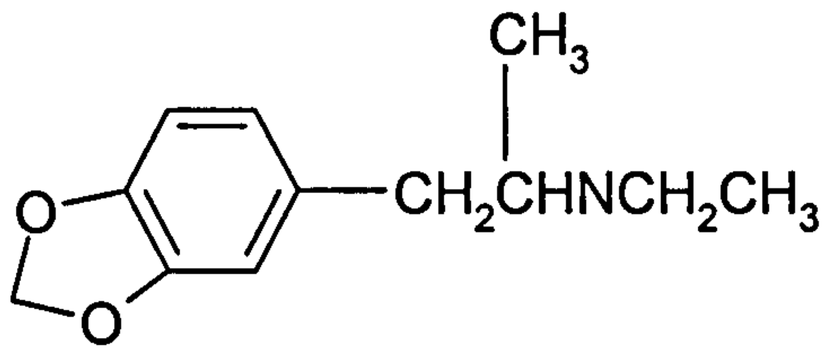
MA



MDA



MDMA



MDEA

Figure 3.2 The structure of Amfetamine($C_9H_{13}N$), Methamfetamine($C_{10}H_{15}N$), MDA($C_{10}H_{13}NO_2$), MDMA($C_{11}H_{15}NO_2$) and MDEA($C_{12}H_{17}NO_2$)

Table 3.1 Properties of Amfetamines [58]

Drug	Street Name	MW	PK_a
AF	Bennies, speed	135.2	9.9
MA	Speed, ice, crystal, crank, meth, go, oxblood	149.2	10.1
MDA	Speed, love pill	179.2	~10
MDMA	ecstasy, XTC, Adam, M&M, MDM, E	193.2	
MDEA	Eve	207.3	

Enantiomers of Amfetamines

AF, MA and all of methylenedioxy derivatives of amfetamine drugs contain a chiral centre and they have a pair of enantiomers. There are two possible enantiomers for these compounds, d and l enantiomers designated as S-(+) and R-(-), respectively[75].

The enantiomers show differences in their pharmacology and toxicology such as metabolism rate, half-life, psychomotor performance and body disposition [55, 76]. For example, the (S)-(+)-isomers of AF and MA have five times more potency as CNS stimulants than the (R)-(-)-isomer. The (S)-isomers of MDMA and MDE are responsible for the psychostimulant and entactogenic activities as compared with the hallucinogenic properties of the (R)-isomers [67, 76]. It has been reported that most of the immunoassay and confirmation tests used can not discriminate between the two different enantiomers [67]

AF and MA compounds are also available by prescription or over the counter in certain countries. For example in the United States, a Vicks Nasal inhaler contains the (R)-(-)-methamfetamine enantiomer. Therefore, the determination of the individual enantiomers is important and may play a role in determination of the potential source of AF or MA [74].

3.1.3 Amfetamine and Methamfetamine Precursor Compounds

There are difficulties in the interpretation of AF and MA positive results in biological samples in clinical and forensic toxicology. These difficulties are related to at least 14 compounds which are precursors of AF and MA. These precursors can be converted metabolically to AF and MA or both. A list of precursors is shown in Table 3.2. To avoid misinterpretation of results and distinguish between an illegal or legitimate (prescription) use, it is essential to consider the specific identification of metabolites and/or unchanged precursor drugs [74].

There are other precursor sources that may be converted to AF such as the monoamine oxidase inhibitor tranylcypromine and 1-phenyl-2-nitropropane. Further tests are needed to prove or exclude that this is the case for these compounds [64]. There are no legal precursor drugs for the "designer" amfetamines [75]. Consequently AF and MA positive results produced from preliminary screening (immunological identification) must be further investigated to identify the cause of the positive preliminary screening results to discriminate between an illegal or legitimate use [77].

Table 3.2 Precursor Compounds to Amfetamine and Methamfetamine

Drug name (Brand Name)	Metabolites in urine [78]
Amphetaminil	AF
Benzphetamine	MA, AF, Others
Clobenzorex	AF, Others
Deprenyl(selegiline)	MA, AF
Dimethylamfetamine	MA, AF, Other
Ethylamfetamine	AF, Other
Famprofazone	MA, AF, Other
Fencamine	MA, AF
Fenethylline	AF, Others
Fenproporex	AF
Furfenorex	MA, AF, Others
Mefenorex	AF, Other
Mesocarb	AF, Others
Prenylamine	AF, Others

3.1.4 Toxicity

Toxicity is an unwanted response to drug use that can produce undesirable side effects and at worst may lead to fatal poisoning. It is necessary to understand the pharmacological and pharmacokinetic properties of drugs to predict their toxicity [58]. Chronic AF use increases its toxicity and can cause mild, moderate or severe toxicity such as vomiting, confusion and coma respectively. Other effects include neurotoxicity and may lead to impairment of brain function [55].

When used therapeutically, AF blood concentrations are usually below 0.1μg/mL. In deaths from acute poisoning, when used alone, measured concentrations in peripheral blood are generally above 2μg/mL. Prescribed doses are up to 40 mg daily whereas when abused, the dose is estimated to be up to 2g.

For MA, the therapeutic blood concentration is generally below 0.1μg/mL. In fatalities when used alone, the measured concentration in peripheral blood has ranged between 0.09 - 18 μg/mL. When abused the dose can be up to 2g [58, 79].

Consumption of methylenedioxy derivatives of amfetamine can cause fatal poisoning. An overdose can give rise to hallucinations, coma and may lead to death [80, 81]. Doses of between 50 – 250 milligrams of MDA can result in blood concentrations up to 0.4 mg/L. Consumption of between 100 – 150 milligrams of MDMA has resulted in blood

concentrations up to 0.3mg/L. In fatalities, concentrations of greater than 0.1mg/L have been measured in peripheral blood for traffic accident victims [58]. MDMA abuse may cause acute and severe toxic effects leading to complications and undesirable side-effects such as hyperthermia, hepatotoxicity and may cause death [82]. MDMA administration produced different effects such as mental and behavioural effects compared to other amfetamine compounds [83]. It is reported that there are associations between death during dancing and MDMA abuse, where a group of young adults died after ingestion of MDA and MDMA during their rave dancing [68]. MDEA has similar psychoactive effects to MDMA but at equivalent doses has less neurotoxic effects [55]. The dose abused is estimated to be between 30 and 100 mg [4].

Blood concentrations of AF and related compounds found in fatal cases either alone, with alcohol or other drugs are shown in Table 3.3 [84]. Also the concentrations of AF and related compounds found in related deaths in various biological matrices are displayed in Table 3.4 [85].

Table 3.3 Fatal amfetamines and related compounds concentrations in blood

Compound	Blood Concentration (mg/L)		
	Alone	With Alcohol (EtOH)	With other drugs
AF	0.1-2.6 n = 5	0.1-0.75 n = 5	0.02-0.64 n = 12
MA	0.35 n = 1	n/a	n/a
MDA	0.04-0.05 n = 2	0.12-0.30 n = 3	0.15-1.2 n = 4
MDMA	0.23 n = 2	0.17-0.60 n = 3	0.03-8 n = 5
MDEA	0.77 n = 1	0.22-2 n = 3	2.44-4.32 n = 2

n/a: not available

Table 3.4 Fatal amfetamine and related compounds concentrations in different biological matrices

Compound	Urine (mg/L)	Liver (mg/kg)	Kidney (mg/kg)	Brain (mg/kg)	Stomach Contents	Muscle (mg/kg)
AF	33.4-700 n = 4	11.7-45 n = 8	3.9-48 n = 5	3-41 n = 3	21mg n = 1 3000 mg/kg n = 1	4 n = 1
MA	1-398.4 n = 10	0.6-174.7 n = 19	0.2-87 n = 17	0.2-101.8 n = 17	5.8-44 mg n = 4 17-514.3 mg/L	3.6-47.7 n = 6
MDA	168-175 n = 2	11-13 n = 2	18 n = 1	10 n = 1	0.25-1.7 mg n = 2	n/a
MDMA	14.3-529 n = 3	6.4-39.7 n = 4	12.1 n = 1	13.7-17.4 n = 2	96 mg/L n = 1 118 mg/kg n = 1	4.5 n = 1
MDEA	201 n = 1	19 n = 1	15 n = 1	28 n = 1	52 mg/kg n = 1	n/a

n/a: not available

3.1.5 Disposition in the Body

Physical and chemical properties of drugs play a major role in their absorption, distribution and elimination [55].

3.1.5.1 Absorption

There are different routes of amfetamines administration (swallowed or injected) but, AF itself is usually consumed orally either as the (S)-(+)-enantiomer (dexamfetamine) or the racemic mixture (eg. amfetamine sulphate). The tablet form of AF is the most commonly encountered amongst abusers. Amfetamines are rapidly and readily absorbed following ingestion. After a single oral administration they reach their plasma concentration peak within 4 hours. No significant differences between racemic and individual isomers were observed in pharmacokinetic parameters (Cmax and Tmax) during the absorption phase.

While, the general routes of MA administration include the oral route, they can be injected intravenously, snorted ('speed') or can be smoked as (S)-(+) - methamfetamine hydrochloride ('ice', vapour inhalation) through a glass pipe system. It was observed that the plasma concentration of smoked MA was higher than its concentration if taken by the oral administration; bioavailability was 90% and 67% respectively. Tmax values of smoked and intravenous MA administration were shorter than the oral administration. 1-2.5 hours, these for smoked and intravenous and at least 3 hours after oral administration.

Methylenedioxy derivatives are mostly consumed orally and are absorbed into the blood after administration. Pharmacokinetic properties of MDEA are similar to those of MDMA when given orally [55]. With a typical dose (60–120 mg), MDA is more potent than MDMA and MDEA. The onset of effects is from 30 to 60 min for MDA and 30 min for MDMA and MDEA and the duration of action of MDA, MDMA and MDEA is about 8 h, 6 h and 3–4 h respectively [68].

3.1.5.2 Distribution

AF and related compounds have relatively low molecular weights, are weak bases and have a low protein binding (around 20%). These parameters enhance their diffusion across cell membranes, lipid layers and tissues, consequently, increasing their accumulation and concentration in different compartments such as tears, saliva, sweat, hair and nails. Therefore, it is potentially one of the best drug groups suitable for testing in these

biological samples. It was noted that there was no difference in the protein binding and distribution volumes of AF enantiomers (S & R), while, the AF (S) enantiomer exhibits a faster metabolism than the (R) enantiomer. Consequently, the elimination half-life will be shorter for (S) enantiomer. There are similarities between volume of distribution of MA and AF. Concurrent use of ethanol will cause a decrease in the volume of distribution of MA due to ethanol displacing MA from peripheral binding. There are limitations in the distribution of designer amfetamines in humans after administration. The passage of AF and related compounds from blood to other fluids is regulated and depends on pKa and the pH in each biological fluid. For example, AF tends to accumulate in saliva and sweat, due to their pH being lower than plasma pH (7.4). It was noted that after oral ingestion, the AF concentration in oral fluid was higher than its concentration in plasma [55].

3.1.5.3 Metabolism

3.1.5.3.1 Amfetamine

P–hydroxyamfetamine and benzoic acid including its glycine conjugate are the main metabolites of AF in the body. Also, unchanged AF can be detected in urine [86]. AF is metabolised after absorption whereas, under normal conditions a large fraction is inactivated by virtue of being deaminated to phenylacetone, then oxidized to benzoic acid and excreted as conjugates. A small fraction of AF is converted by oxidation to norephedrine. Norephedrine and its precursor are p–hydroxylated [79]. The metabolic pathway of AF is shown in Figure 3.3.

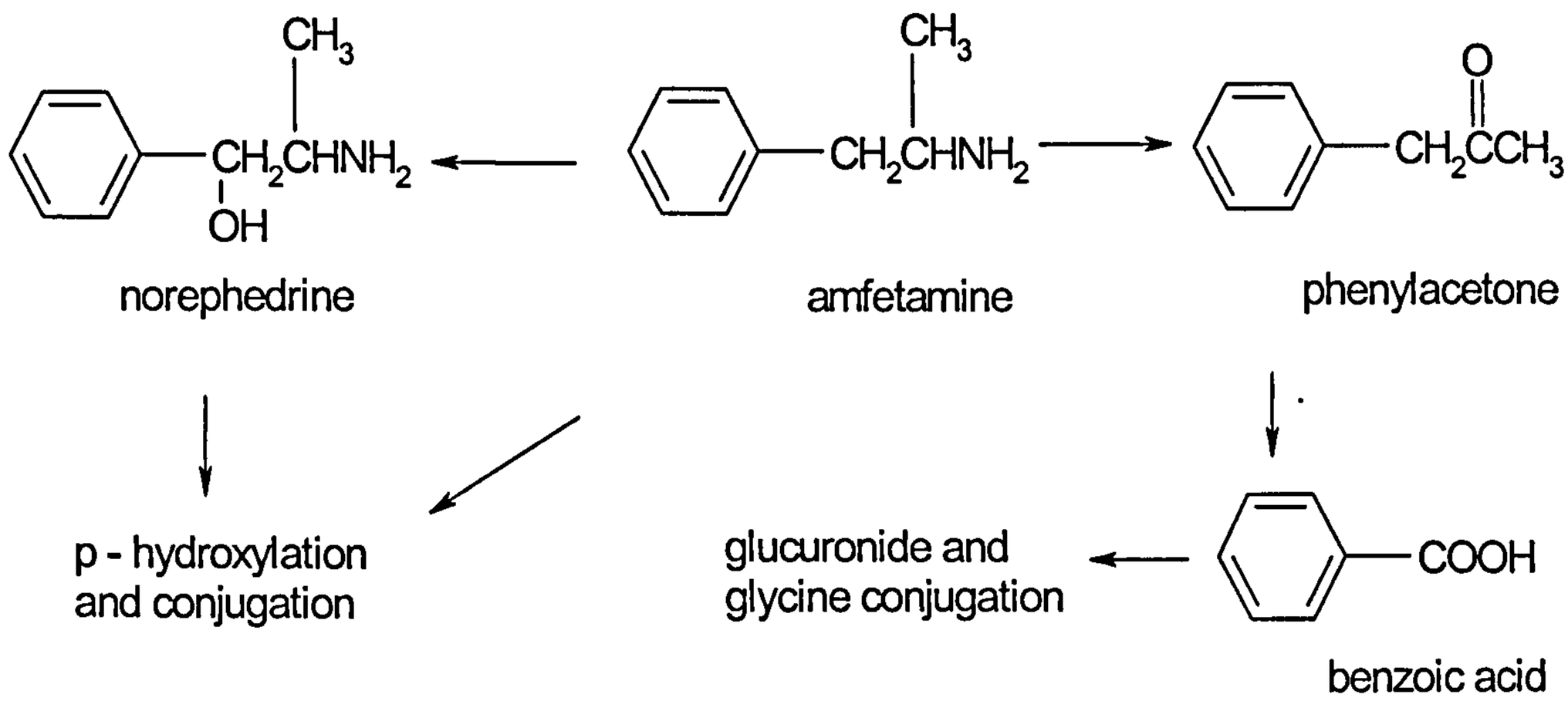


Figure 3.3 The metabolic pathway of amfetamine in human body [79]

3.1.5.3.2 Methamfetamine

The N- demethylated form of methamfetamine produces amfetamine as an active metabolite [79]. The other metabolic pathways of MA when converted to p–hydroxylated metabolites and benzoic acid are the same as for AF metabolism, but with a lower formation of benzoic acid than from AF [86]. The metabolic pathway of methamfetamine is shown in Figure 3.4.

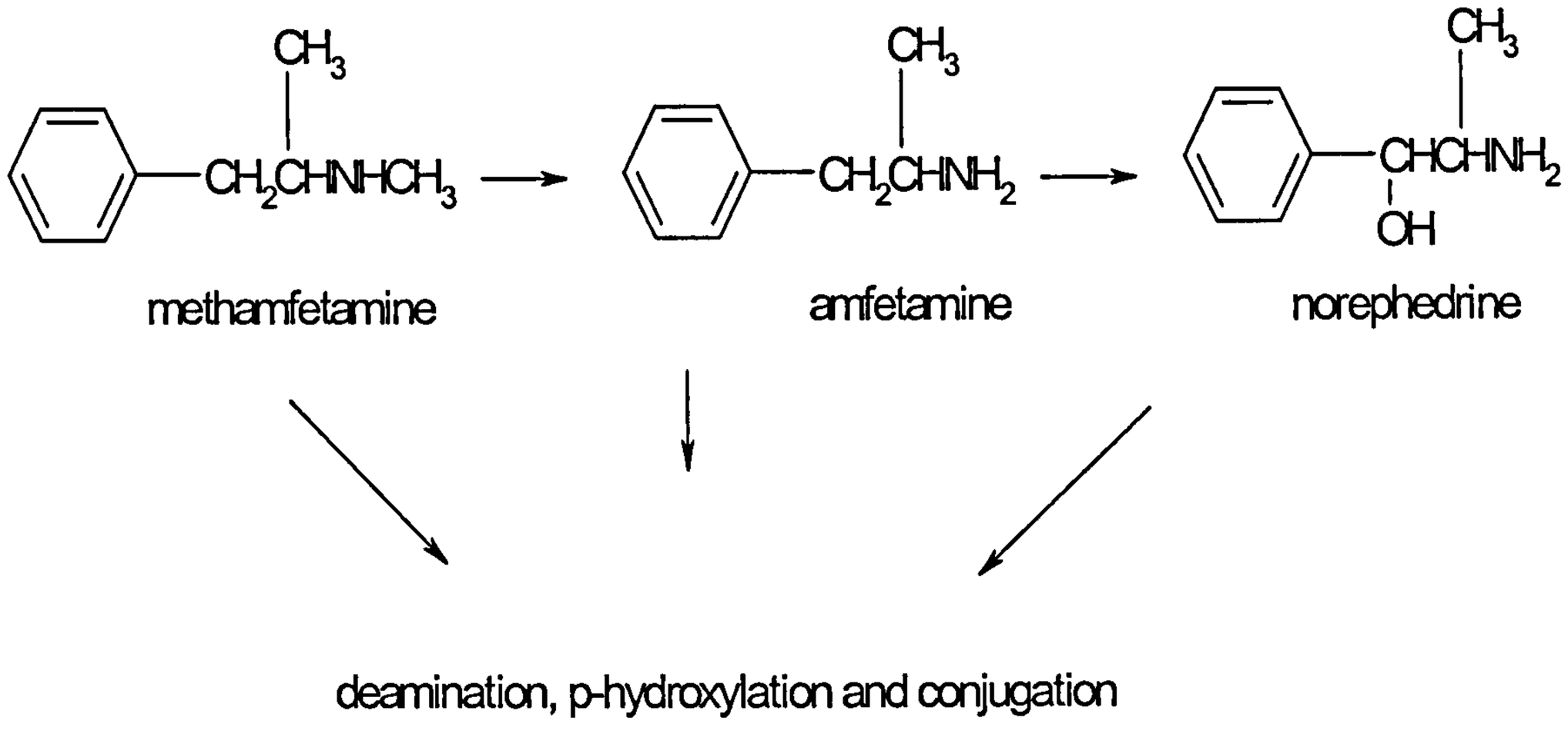


Figure 3.4 The metabolic pathways of methamfetamine in human body [79]

3.1.5.3.3 Methylenedioxy derivatives

Extensive metabolism is carried out for methylenedioxy derivatives of amfetamine. Monohydroxy and dihydroxy metabolites are formed [55, 67]. MDA and other metabolites are produced from metabolism of MDMA and MDEA [58]. The metabolic pathways of MDMA and MDEA are shown in Figure 3.5 and Figure 3.6. Limited information is available for methylenedioxyamfetamine (MDA) metabolism in humans.

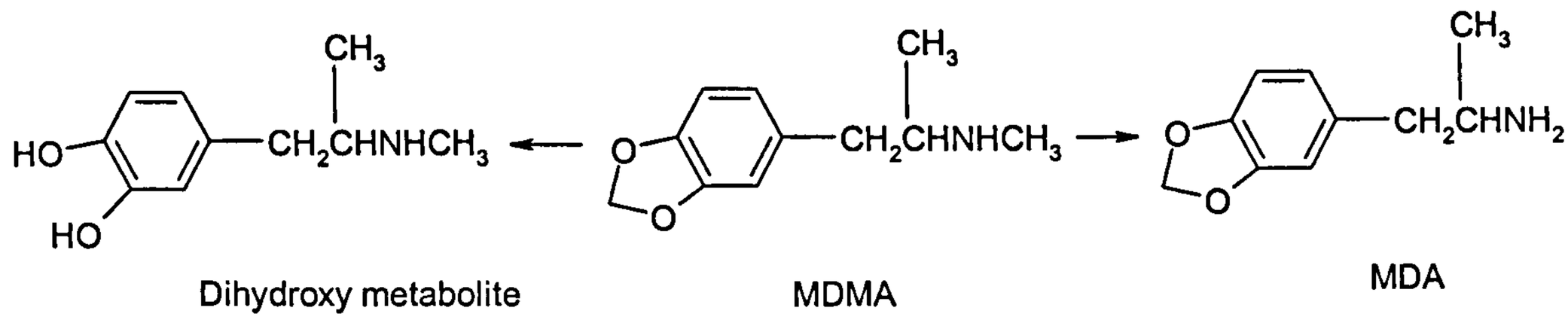


Figure 3.5 The metabolic pathways of MDMA in human body [58]

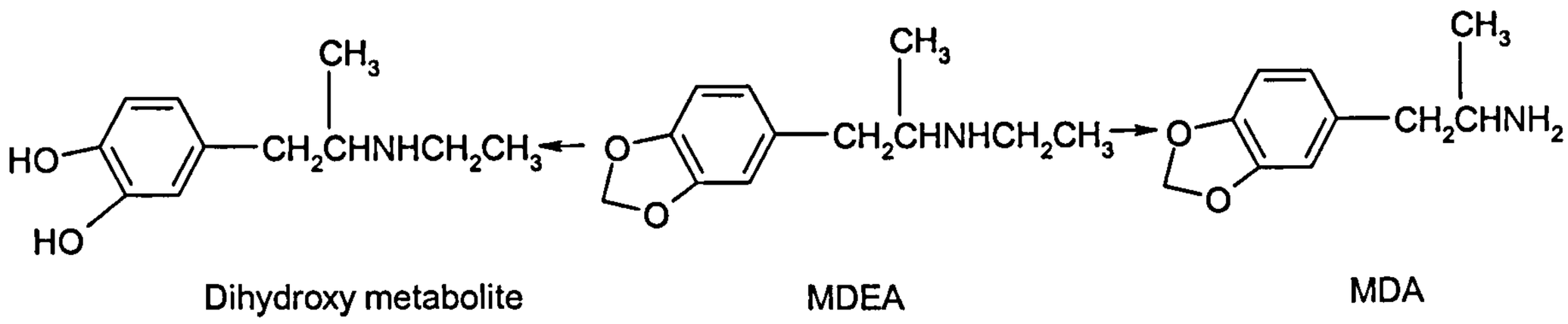


Figure 3.6 The metabolic pathways of MDEA in the human body [58]

3.1.5.4 Excretion

Excretion of AF and related compounds is pH dependent. Due to their weak basic properties, urinary acidification increases their excretion and urinary alkalination decreases it. Consequently, a significant variation of plasma half-life is exhibited. The values are shorter in urinary acidification and longer in urinary alkalination. It was observed that the excretion of unchanged MA decreased with increasing doses. Smaller amounts of unchanged methylenedioxy derivatives were excreted in urine than with any of the other amfetamine compounds [55]. Only a small amount of AF was reabsorbed under acid conditions, while, more AF was reabsorbed under alkaline conditions. This may explain the increase and decrease of AF excretion [60]. 30% of AF excreted was unchanged in 24 hour urine. This increased to 74% with acidic urine and decreased to 1% in alkaline urine. 43% of MA was excreted unchanged in a 24 hour urine sample. A further 4-7% was recovered as AF. In acidic and alkaline urine, these percentages were changed. Whereas 76% and 2% of MA was recovered unchanged in the acidic and alkaline urines respectively, the corresponding recoveries for AF were approximately 2% and less than 0.1% respectively. 26% of MDMA was excreted unchanged in 24 hour urine and 1% as MDA after single oral dose administration (100-125 mg). 19% of MDEA was excreted unchanged in 32 hour urine and 28% as MDA after single oral dose administration [79].

3.1.6 Hair Analysis for Amfetamines

There are many different validated analytical methods containing a variety of decontamination, pre-treatment, extraction, derivatisation procedures and instrumental

techniques which are all suitable for amfetamines detection and have been used for amfetamines analysis in hair [87-112].

The most routine decontamination methods used for hair containing amfetamines are acid, methanol, 0.1 % sodium dodecyl sulphate (SDS) with water, water with acetone and dichloromethane [97]. 0.1 % SDS has been used to decontaminate hair soaked in an aqueous solution of 10 μg/ml of MA hydrochloride for 24 h [19]. This was shown to easily and effectively remove this external contamination. A review of the literature showed that 0.1 % SDS and water have been used frequently as wash solvents for MA and its metabolites in hair [113].

Several pre-treatment methods have been used to extract drugs from the hair. These have included alkaline, acid, enzymatic and methanol pre-treatments. A GC-MS method has been reported for the detection of AF, MA, MDA and MDMA simultaneously in hair [93]. This involved digesting the hair using 1 M sodium hydroxide followed by LLE and derivatisation with pentafluoro-propionic anhydride / pentafluoropropanol. In this case the limits of detection were 0.05 ng /mg for AF, MA and MDA and 0.1 ng/ mg for MDMA. This method showed high specificity and sensitivity for AF, MA, MDA and MDMA in hair samples.

Mild enzymatic treatment (Proteinase K) with Tris buffer at a pH of 6.2 was reported to give good recoveries by releasing amfetamines, cannabinoids and others drugs from hair by using mass spectrometric analysis (GC-MS) [28]. An advantage of enzymatic treatment is that it is more suitable for certain unstable drugs compared with other pre-treatment methods such as alkaline pre-treatment [19]. A GC-MS method was developed for the simultaneous analysis of amfetamines and other compounds such as opiates and cocaine in hair using β-glucuronidase / aryl-sulfatase as pre-treatment method. This was followed by SPE and derivatisation with pentafluoro-propionic anhydride / pentafluoropropanol. The limits of detection for this method were less than 0.1 ng/mg for all substances and the AF recovery was 50% for spiked control hair [95].

Methanolic extraction with sonication was used by another group to extract AF and its methylenedioxy-derivatives (MDA, MDMA and MDEA) simultaneously from hair. Propionic acid anhydride (PSA) or trifluoroacetic acid anhydride (TFA) derivatisation reagents are used. GC-MS was used in selected ion monitoring mode for the detection. The limits of detection were 0.01 ng/mg for all compounds. This method proved that

methanolic extraction with sonication could be used successfully to extract amfetamines simultaneously with other drugs such as opiates [91].

Two studies which compared various extraction methods for the recovery of amfetamines have been reported. The first study showed that alkaline pre-treatment produced the highest recoveries when followed by LLE. The second study showed that acid pre-treatment produced the highest recoveries when followed by LLE and then SPE [92, 94].

Many procedures used for the extraction of amfetamines from hair samples are similar to those used for blood analysis of amfetamines. Bond-Elut Certify SPE columns have been shown to give clean extracts from whole blood for AF and MA [67]. AF, MA, MDA, MDEA and MDEA were successfully extracted from hair using Isolute® Confirm HCX-3 (130 mg) SPE. The recoveries were greater than 70% for all five amfetamines [114]. LLE methods and SPE methods have both been used [67, 75, 93, 95, 114].

Derivatisation agents that have been successfully used for derivatisation of amfetamines following extraction of amfetamines from hair are typical of those used routinely for the derivatisation of amfetamines. These include trifluoroacetic anhydride (TFA), chlorodifluoroacetic anhydride (CDFA), heptafluoro-n-butyryl (HFB), pentafluoro-propionic anhydride (PFPA), N-methyl-bis-trifluoroacetamide (MBTFA) pentafluoro-propionic anhydride / pentafluoropropanol (PFPA / PFPOH) and propionic acid anhydride (PSA) [91, 93, 113].

A number of analytical methods have been reported for screening AF and related compounds in biological samples. These qualifying techniques have included: immunoassay techniques such as enzyme multiplied immunoassay technique (EMIT), radioimmunoassays (RIA), enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA) and fluorescence polarization immunoassay (FPIA) [57, 115-117]. Immunoassay has been used for screening of amfetamines in hair. These types of technique have included EMIT [118] and ELISA [119].

The confirmation techniques for amfetamines in biological specimens have included: gas chromatography–mass spectrometry (GC-MS), gas chromatography tandem mass spectrometry (GC-MS-MS), in electron impact, positive and negative chemical ionisation modes, GC with flame-ionisation detection (GC-FID), nitrogen–phosphorus detection (GC-NPD) or electron-capture detection (GC-ECD) and high-performance liquid chromatography with ultraviolet detection (HPLC-UV) or chemiluminescence detection

(HPLC-CD), capillary electrophoresis (CE), liquid chromatography mass spectrometry (LC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS-MS) [19, 115, 120, 121]. GC-MS has been frequently used for the identification of amfetamines and it is the most common method used for identification of amfetamines in hair [67, 91, 93, 95].

3.2 Cannabis

3.2.1 Introduction

The cannabis sativa L. plant which includes its sub-species (hemp plant, variety Indica) is the source of cannabis (marijuana and hashish). These are dioecious and perennial plants. They contain over 420 unique chemical compounds of which at least 61 compounds are cannbinoids [122]. The female plants produce more of the main active constituent Δ^9- THC than male plants. The commonly used "street" names of cannabis and its products are weed, grass, dope, dagga and pot [58]. The major cannabinoid substances in the cannabis plant are Δ^9- THC, cannabidiol (CBD) and cannabinol (CBN). The main psychoactive agent identified in the plant is Δ^9- THC which was first isolated in the 1960. Its acid precursor, Δ^9 –tetrahydrocannabinolic acid (Δ^9-THCA) is not psychoactive. It does, however, account for most of the THC related substances present in the plant. Only a small amount is present as neutral THC [123]. Δ^9 –THCA is converted to active Δ^9 – THC by heat or when cannabis products are smoked (decarboxylated) [124]. In addition, it was shown only that Δ^8 –tetrahydrocannabinol (Δ^8 -THC), Δ^9 –tetrahydrocannabivarol and CBN have significant psychoactive properties [125].

Since ancient times, the cannabis plant and its product were used for recreational, medicinal and religious purposes in different cultures [126]. Also, it is used as a fibre source (hemp) [127]. In the beginning, marijuana was taken by the inhalation of volatile oils generated in the smoke. There are reported usages for a number medical conditions including pain, anxiety, glaucoma, nausea (for cancer chemotherapy), emesis, muscle spasms and wasting diseases [128]. Dronabinol and Marinol are prescription forms for Δ^9-THC [79]. Δ^9- THC is often present at low concentration with no psychoactive effects in shampoo preparations, in foodstuffs (product) such as oil, noodles, crackers and in beverages such as tea [129]. Cannabis material has been used in food products in different global markets as nutritional supplements in the last few years [130]. Due to its sedative and anxiolytic properties, it has been used by athletes to reduce stress [131].

The use of a high dose of cannabis can cause euphoria and relaxation followed by a depressant period [132]. Cannabis is available in various preparations. The concentration of Δ^9 -THC depends on the particular preparation and formulation type. Marijuana,

hashish, oil are prepared from dried plant (herbal cannabis), resin (cannabis resin) and by distillation respectively. It is usually mixed with tobacco and is rarely chewed [133]. It is reportedly taken with other drugs such as alcohol to enhance the psychoactive effect [132].

The administration route of cannabis can be either oral, such as marijuana mixed with food, e.g. brownies, or by inhalation (smoking) [134]. Overall, the main administration route is smoking [13]. Cannabis is the most commonly and frequently used illegal substance worldwide and recent studies demonstrated that about one to two thirds of young people in Australia, Germany, New Zealand and the United Kingdom have tried cannabis at least once prior to young adulthood [135]. It ranked next to alcohol and nicotine [136]. In accordance with European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), Europe, 2001, Substance Abuse and Mental Health Services Administration (SAMHSA), USA, 2001 and Australian Institute of Health and Welfare, Australia 2002, cannabis is the most widely abused drugs in these countries [137]. It was reported that cannabis use has the potential risk of leading to the usage of other harder illegal drugs [138]. It was reported in 2004 that there are approximately 300-400 million consumers of cannabis preparations in the world [122]. According to the BCS, cannabis was the most commonly used drug in 2004/5. It was estimated that 23.5% of 16 to 24 year olds in England and Wales used this drug in the last year [3].

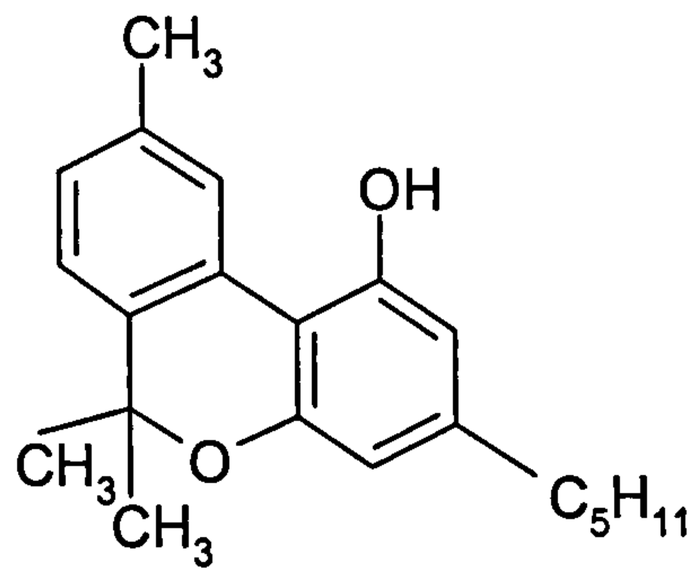
An association between cannabis consumption and psychosis has been reported [136]. Its biological effect is initiated by its interaction with cannabinoid receptors such as CB1 and CB2 which are found in mammalian tissue and widely distributed in the CNS and peripheral tissue [139]. There are various effects of cannabis, either acute effects such as loss of internal control and impairment of attention and memory especially, during intoxication and adverse chronic effects on cognition. This may cause cannabis dependence syndrome which is an inability to control or to abstain from cannabis use [127]. It was confirmed as a risk factor for arthritis and it is reported that intoxication induces aggressive behavior [133]. Dry mouth, blurred vision and ataxia (an increased heart rate) can be attributable to Δ^9 –THC use [140].

It has been reported that one in 11 cannabis users will become dependent. Cannabis withdrawal was characterised by different signs and symptoms such as irritability, anxiety, sleep disruptions, aches, and pains, which lead to difficulty in maintaining abstinence. Adverse effects on cognitive function were also reported and observed with heavy cannabis use and it was reported that pulmonary dysfunction was associated with regular cannabis smoking similar to regular tobacco smokers [141]

5–10 mg of cannabis smoked is enough to produce the effects such as euphoria, hallucinations and often sedation [134]. At a federal level in USA, medicinal use of smoked or ingested marijuana is prohibited [128]. Cannabis has been listed since January 2004 in Class C of the Misuse of Drugs Act 1971 [3].

3.2.2 Chemistry

The main active component of cannabis is Δ^9 –THC, which exists in a variety of stereochemical forms. These include (-)-trans-Δ^9-tetrahydrocannabinol isomer, also known as dronabinol or Δ^9 –THC. There are other cannabinoid compound isomers which have less potential pharmacological activity than Δ^9 –THC for instance Δ^8 –THC [140]. The chemical formula of Δ^9 –THC is $C_{21}H_{30}O_2$, its molecular weight is 314.5 and its pKa is 10.6. It is insoluble in water [4]. The chemical structure of Δ^9 –THC is shown in Figure 3.7.



THC

Δ^9 –tetrahydrocannabinol (Δ^9 -THC)

Figure 3.7 Chemical structures of Δ^9-THC

3.2.3 Disposition in the Body

3.2.3.1 Absorption and Distribution

Δ^9 –THC and other cannabinoid compounds are absorbed by inhalation (smoking) or by ingestion (orally). The concentration of its metabolites will depend on the route of administration [58]. Δ^9 –THC is distributed widely in the body to fat and muscle and its concentrations in blood decline due to its lipophilic and low water solubility properties. Within 15 min of smoking, the peak plasma concentrations of Δ^9 –THC may exceed 50 ng/ml and reach 200 ng/ml with higher THC doses [125].

3.2.3.2 Metabolism

Δ^9 –THC is rapidly oxidized to the psychoactive metabolite 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), which is subsequently further oxidized to the non-psychoactive metabolite Δ^9 -THC-COOH. Minor metabolic pathways are carried out to produce smaller quantities of the other metabolites 8-alpha-OH-THC and 8, 11-dihydroxy-THC. Δ^9-THC and its hydroxylated and carboxylated metabolites are conjugated with glucuronic acid, and are subsequently excreted in urine as water soluble compounds [79, 130, 142]. The metabolism of Δ^9-THC is shown in Figure 3.8.

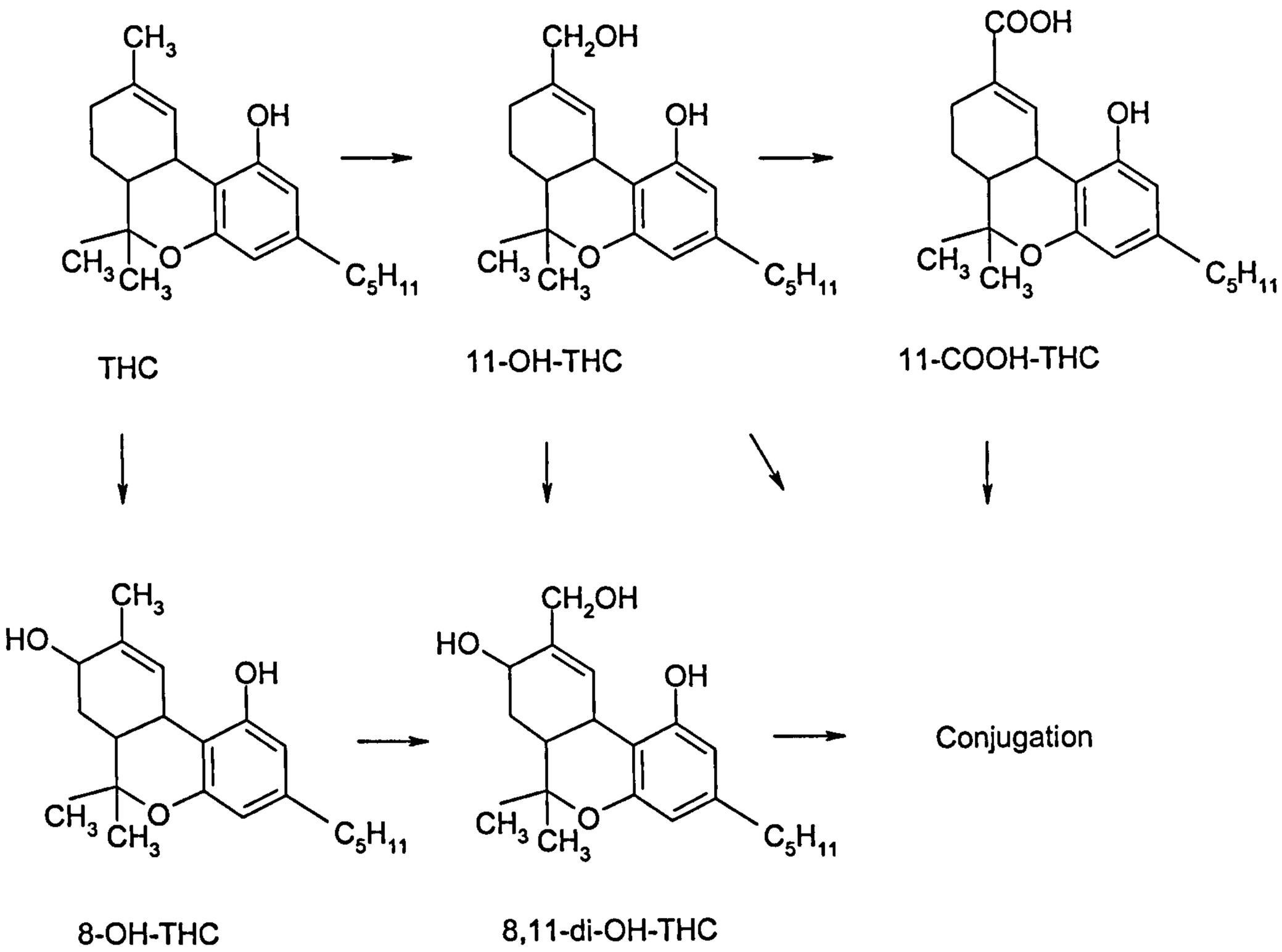


Figure 3.8 Δ^9-THC and Δ^9-THC-COOH Metabolic Pathway [79]

3.2.3.3 Excretion

Often cannabinoids and their metabolites are excreted as glucuronic acid conjugates, therefore require hydrolysis prior to analysis [142]. Conjugated and unconjugated Δ^9 -THC-COOH are the major metabolits found in urine samples. Δ^9-THC and its metabolites Δ^9 -THC-COOH and 11-OH-THC are found in blood. The main compound in hair is Δ^9-THC [13]. Within 72 hours, 70% of Δ^9-THC dose is excreted. Approximately 40% in the faeces and 30% in the urine, A small amount of unchanged Δ^9-THC is excreted in urine [79]. It is reported that the urinary excretion half-life of Δ^9-THC is 3–13 days and that of the main urinary metabolite Δ^9 -THC-COOH is 12 -25 days, depending on frequency of use [125].

It is reported that only Δ^9-THC is detectable in saliva and sweat. The other metabolites are rare. CBN, THC, CBD and Δ^9 -THC-COOH are detectable in hair, but Δ^9 -THC-COOH is at very low concentrations, while in meconium only Δ^9 -THC-COOH is detectable [143].

3.2.4 Hair Analysis for Cannabinoids

A number of methods have been described regarding cannabinoids in hair samples [122, 129, 134, 144-157]. The first method to determine Δ^9 -THC and Δ^9 -THC-COOH in human hair used gas chromatography/mass spectrometry for detection. Dichloromethane was used to decontaminate the hair. This was followed by alkaline pre-treatment using 1 N NaOH, LLE (n-hexane/ethyl acetate) and derivatisation when was carried out using PFPA/PFP-OH. The limit of detection of both Δ^9 -THC and Δ^9 -THC-COOH were 0.1 ng/mg and their recoveries were 75 and 80%, respectively [158].

A sensitive analytical method was developed for quantitative analysis of Δ^9-THC, Δ^9-THC-COOH, CBN and CBD in human hair. Internal standards used were ketamine for CBN and CBD, and ketoprofen for Δ^9-THC and Δ^9-THC-COOH. β –glucuronidase / arylsulfatase was used as pre-treatment method. LLE was used for the extraction and pentafluoropropionic anhydride (PFPA) and pentafluoropropanol (PFPOH) for derivatization. Gas chromatography/mass spectrometry detection, with electron impact ionisation (GC-MS-EI) was used for the analysis of all the compounds. The concentrations of the main metabolite of Δ^9-THC, i.e. Δ^9-THC-COOH are very low in hair. For this reason gas chromatography/mass spectrometry, in negative ion chemical ionisation mode

(GC-MS-NCI) using methane as the reagent gas was used to detect Δ^9-THC-COOH. Extraction recoveries ranged from 80.9 to 104.0% for Δ^9-THC, 76.7–95.8% for CBN, 71.0–94.0% for CBD and 85.9–100.0% for Δ^9-THC-COOH by GC-MS-NCI and 77.7-98.9% by GC-MS-EI. LOD's were 20.00 pg/mg hair for Δ^9-THC and cannabidiol, 50.00 pg/mg hair for cannabinol and 0.50 ng/mg hair for Δ^9-THC-COOH by GC-MS-EI, while Δ^9-THC-COOH by GC-MS-NCI was 5.00 pg/mg hair [153].

The presence of Δ^9-THC-COOH in hair shows conclusive proof of cannabis use and for this reason its identification in hair is important. The finding of the parent drug alone could be as a result of environmental (passive) contamination. Since, the concentrations of the main metabolite of Δ^9-THC, i.e. Δ^9-THC-COOH are very low in hair, high sensitivity techniques, high-volume injection and good clean-up methods are required [122, 153].

4 Analysis of Amfetamines and Cannabinoids

4.1 Aim

The purpose of the following study was to identify amfetamines and cannabinoids simultaneously in hair samples using a single extraction procedure. It was necessary to investigate the pre-treatment steps for all of the compounds of interest to optimize a method for this purpose. Four pre-treatment procedures were chosen (enzymatic, alkaline, acid and methanol) for the comparison study. The optimised method giving the highest recoveries would then be selected to analyse these drugs in hair. The SPE method developed by *Chen et al* using Bond Elut Certify columns has been shown to be successful in extracting a range of drugs (acidic, neutral and basic drugs) in whole blood with good recoveries [159]. This method has also been adapted for the extraction of drugs in meconium [160] and in oral fluid samples [161] and could be used as a clean-up method to extract drugs from pre-treated hair. For this reason this SPE method was selected following pre-treatment by the four different methods. The best pre-treatment method would then be validated.

4.1.1 Experimental

4.1.1.1 Chemicals and Reagents

AF, MA, MDA, MDMA, MDEA, Δ^9- THC and Δ^{9-} THC-COOH and their deuterated internal standards, AF-d_5, MA-d_5, MDA-d_5, MDMA-d_5, MDEA-d_5, Δ^9- THC-d_3 and Δ^9 - THC-COOH-d_3 were purchased from Radian (Teddington,UK). The derivatising reagents pentafluoropropionic anhydride (PFPA) and pentafluoropropanol (PFPOH), β-glucuronidase (activity =86.900 units/ml, sulfatase activity = 2,210 units/ml) and sodium dodecyl sulphate (SDS) were purchased from Sigma (Dorest, UK) and Bond Elut Certify^{TM} from Crawford Scientific (Strathaven, UK). HPLC grade solvents, methanol (MeOH), acetone, chloroform ($CHCl_3$), distilled water (DI H_2O), ethyl acetate (EtOAc), hexane, dichloromethane, analytical-grade ammonium hydroxide, glacial acetic acid, potassium-dihydrogen phosphate, sodium hydroxide (NaOH), hydrochoric acid (HCl) and tartaric acid were obtained from Merck (Poole,UK).

4.1.1.2 Stock Standards

All stock standards had concentrations of 100 µg/ml. These were stored in the freezer at -20 °C. An amfetamine working standard solution was prepared by measuring 50 µl of stock standards of AF, MA, MDA, MDMA and MDEA into a large vial. This was made up to 5 ml with methanol to give a 1 µg/ml combined amfetamines working standard. A cannabinoids working standard solution was prepared by measuring 50 µl of stock standards of Δ^9- THC and Δ^9- THC-COOH into a large vial. This was made up to 5 ml with methanol to give a 1 µg/ml combined cannabinoid working standard. Amfetamine and cannabinoids deuterated internal standard solutions were similarly prepared using AF-d_5, MA-d_5, MDA-d_5, MDMA-d_5, MDEA-d_5, and Δ^9- THC-d_3 and Δ^9- THC-COOH-d_3 to give a combined amfetamine deuterated working standard and a combined cannabinoid deuterated working standard at 1 µg/ml .

4.1.1.3 Instrumentation (GC-MS)

A ThermoQuest Trace GC with a Finnigan Trace MS was used, fitted with a HP-5 capillary column (30 m x 0.32 mm x 0.25 µm film thickness). This was temperature programmed from 100 to 300 °C at a rate of 12 °C / minute and held at 300 °C for 5 min. The injector temperature was 280 °C. The splitless injection mode was used. The carrier gas used was helium. The temperature of the source and interface were 200 °C and 250 °C respectively. The emission current was 350 µV. The injection volume was 1 µl. The ionisation mode was electron impact with an electron energy of 70 eV.

4.1.1.4 Identification of Retention Times and Fragmentation

In order to identify the retention times and mass fragments of each compound and its deuterated standard, 100 µl of amfetamines working standard solution and 100 µl of deuterated amfetamines working standard solution all at 1 µg/ml were measured into a vial along with 100 µl (1 mg/ml) of tartaric acid in ethyl acetate. The solvent was evaporated to dryness under a stream of nitrogen at room temperature and the residue was derivatised using 60 µl of PFPA / EtOAc (0.5:1 v/v) at 60 °C for 30 minutes. This was then evaporated under nitrogen and reconstituted in 30 µl EtOAc, 1 µl of which was injected for analysis by GC-MS.

100 µl of cannabinoids working standard solution and 100 µl of deuterated cannabinoids working standard solution all at 1 µg/ml were measured into a vial. The solvent was

evaporated to dryness under a stream of nitrogen at room temperature and the residue was derivatised using 60 µl of PFPA / PFPOH (1:0.75 v/v) at 60 °C for 30 minutes. This was then evaporated under nitrogen and reconstituted in 30 µl EtOAc, 1 µl of which was injected for analysis by GC-MS.

Derivatising agents which were selected for the above analysis were those that are used routinely in the Forensic Medicine & Science laboratory, University of Glasgow for the identification and measurement of amfetamines and cannabinoids. Analyses were performed using repetitive full scan to identify the retention times and mass fragments of each compound and their deuterated standards.

4.1.1.4.1 Results

The retention times and mass fragments were identified. Good peak shape, good chromatographic resolution and sensitivity were obtained for each compound. The retention times and ions for each compound are shown in Table 4.1. The underlined ions were used for quantitation in further analyses. The chromatograms and mass spectra for each compound are shown in Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5, Figure 4.6, Figure 4.7, Figure 4.8, Figure 4.9, Figure 4.10, Figure 4.11, Figure 4.12, Figure 4.13, Figure 4.14, Figure 4.15, Figure 4.16, Figure 4.17, Figure 4.18, Figure 4.19, Figure 4.20 and Figure 4.21.

Table 4.1 The retention times (t_R), derivatised analyte ions (m/z) and their deuterated standards

Analyte	Retention time (min)	Main Ions (m/z)	Figures
AF	4.34	91,118, <u>190</u>	Figure 4.1 and Figure 4.8
AF-d_5	4.30	<u>194</u>	Figure 4.1 and Figure 4.15
MA	5.52	118,160, <u>204</u>	Figure 4.2 and Figure 4.9
MA-d_5	5.48	<u>208</u>	Figure 4.2 and Figure 4.16
MDA	7.86	135, 162, <u>325</u>	Figure 4.3 and Figure 4.10
MDA-d_5	7.83	<u>330</u>	Figure 4.3 and Figure 4.17
MDMA	8.96	135, 204, <u>339</u>	Figure 4.4 and Figure 4.11
MDMA-d_5	8.93	<u>344</u>	Figure 4.4 and Figure 4.18
MDEA	9.31	190, <u>218</u>, 353	Figure 4.5 and Figure 4.12
MDEA-d_5	9.28	<u>223</u>	Figure 4.5 and Figure 4.19
Δ^9-THC	12.76	<u>377</u>, 417, 460	Figure 4.6 and Figure 4.13
Δ^9-THC -d_3	12.74	<u>380</u>	Figure 4.6 and Figure 4.20
Δ^9-THC-COOH	14.43	445, 459, <u>622</u>	Figure 4.7 and Figure 4.14
Δ^9-THC-COOH-d_3	14.41	<u>625</u>	Figure 4.7 and Figure 4.21

*The underlined ions were used for quantitation

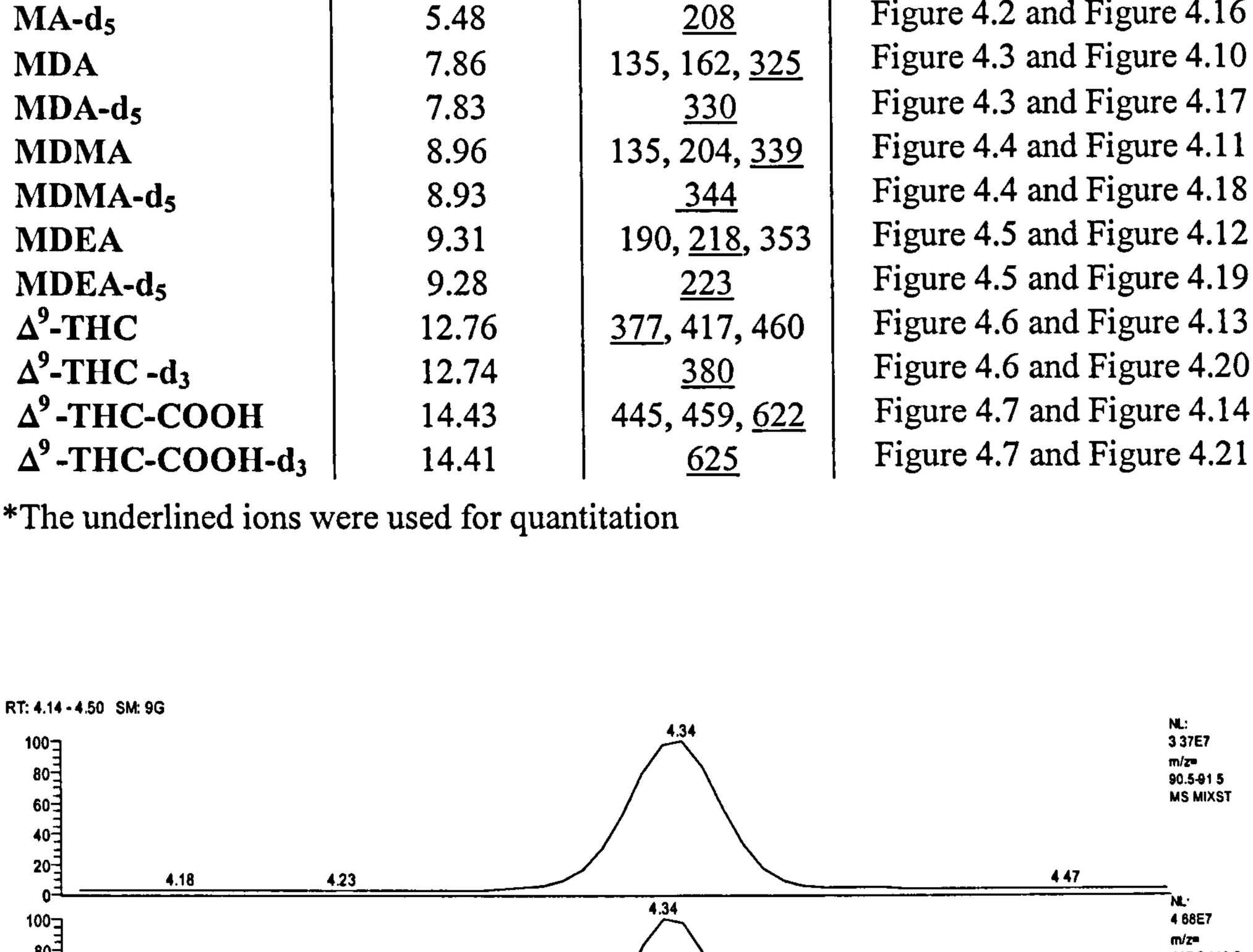


Figure 4.1 GC-MS Chromatograms of PFP derivatives of AF and AF-d_5

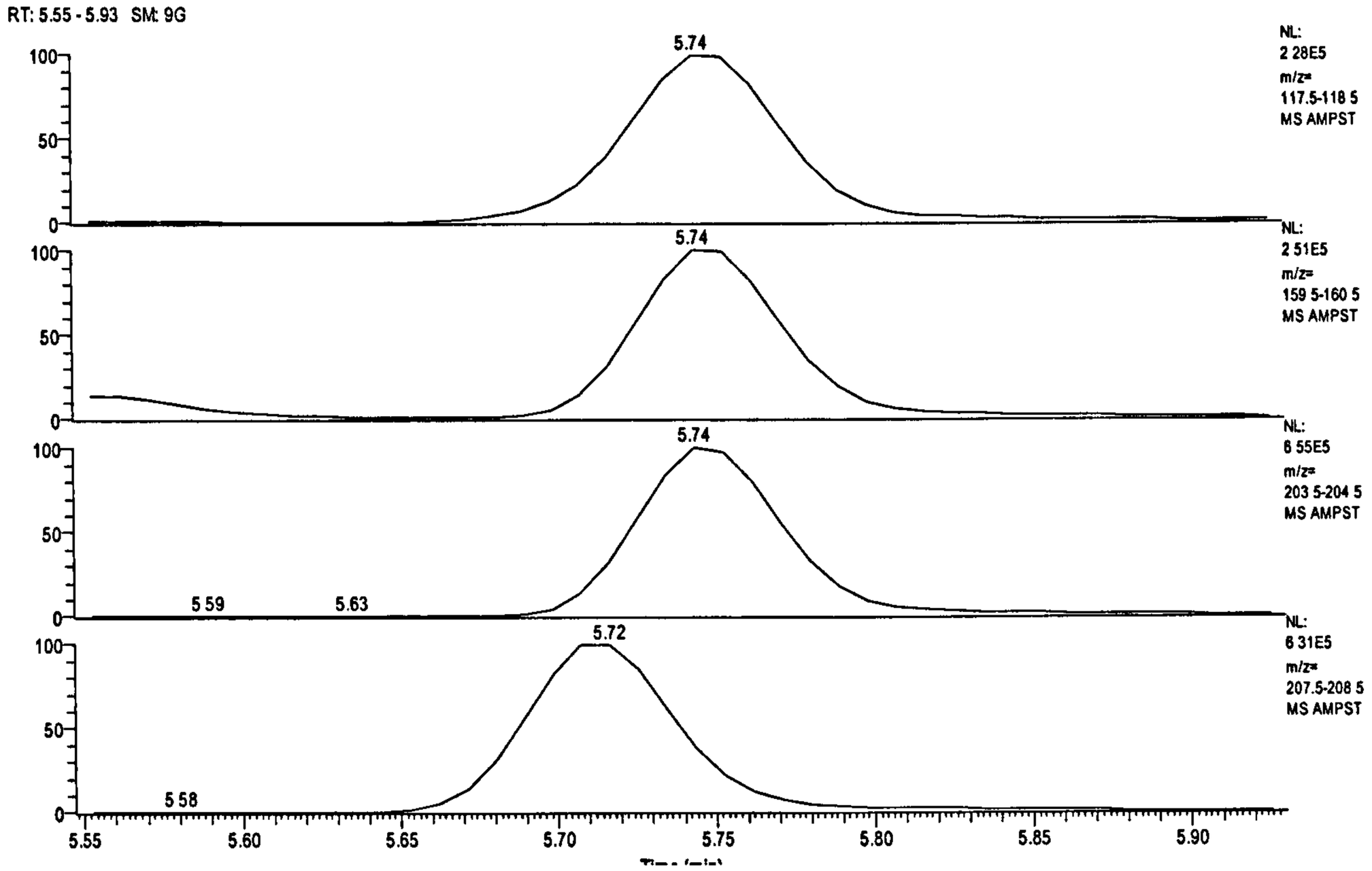


Figure 4.2 GC-MS Chromatograms of PFP derivatives of MA and MA -d_5

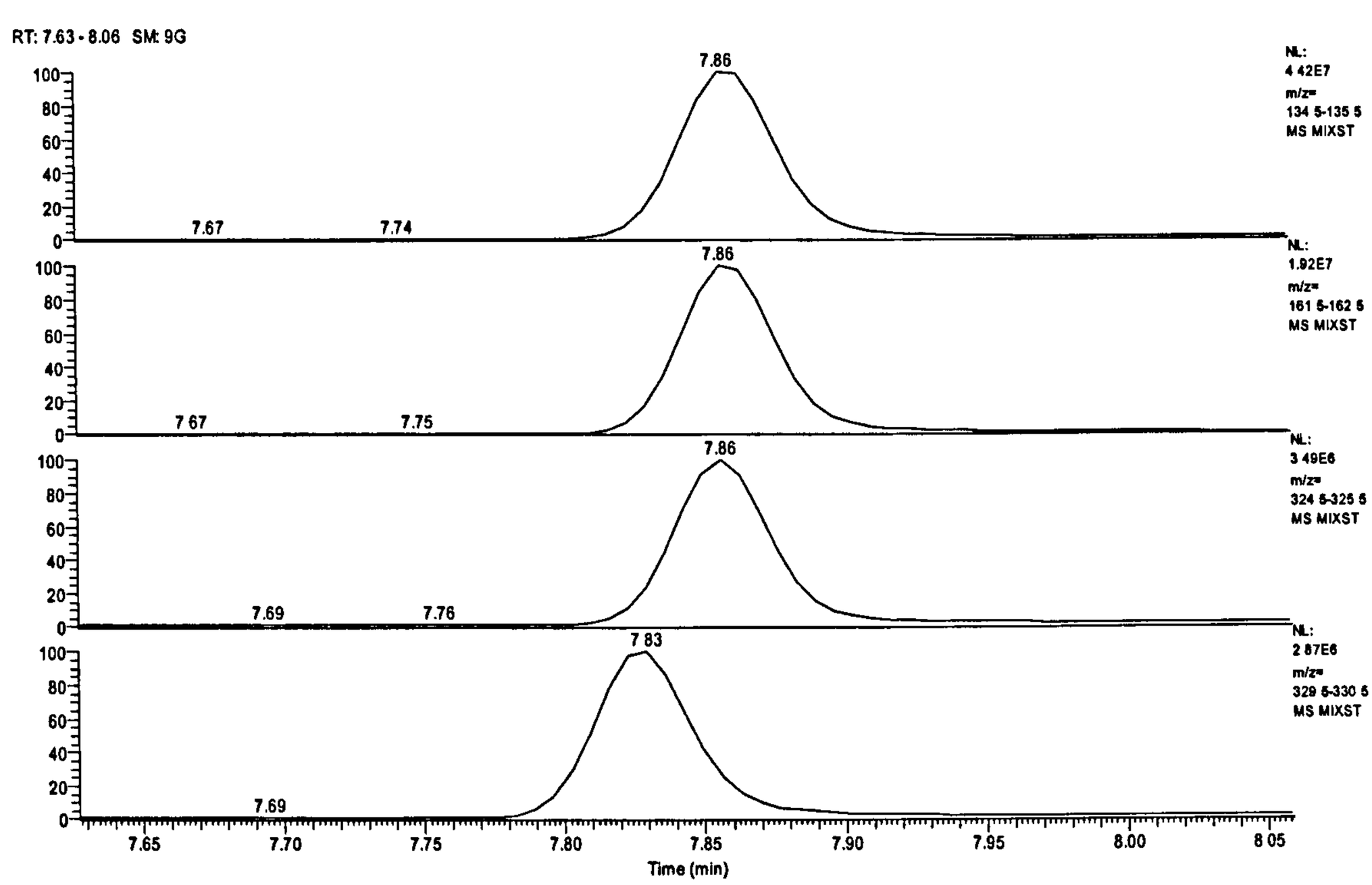


Figure 4.3 GC-MS Chromatograms of PFP derivatives of MDA and MDA-d_5

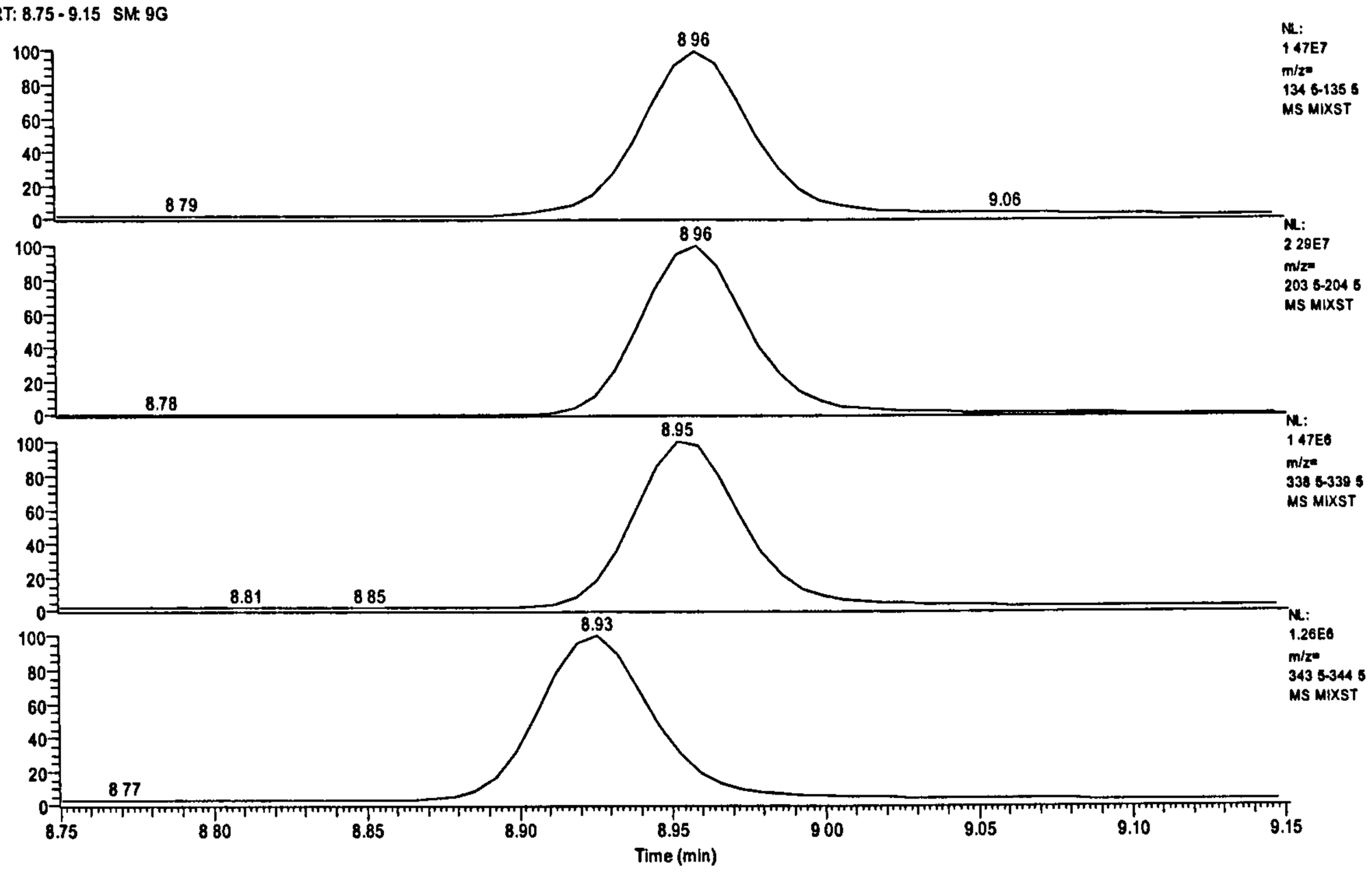


Figure 4.4 GC-MS Chromatograms of PFP derivatives of MDMA and MDMA-d_5

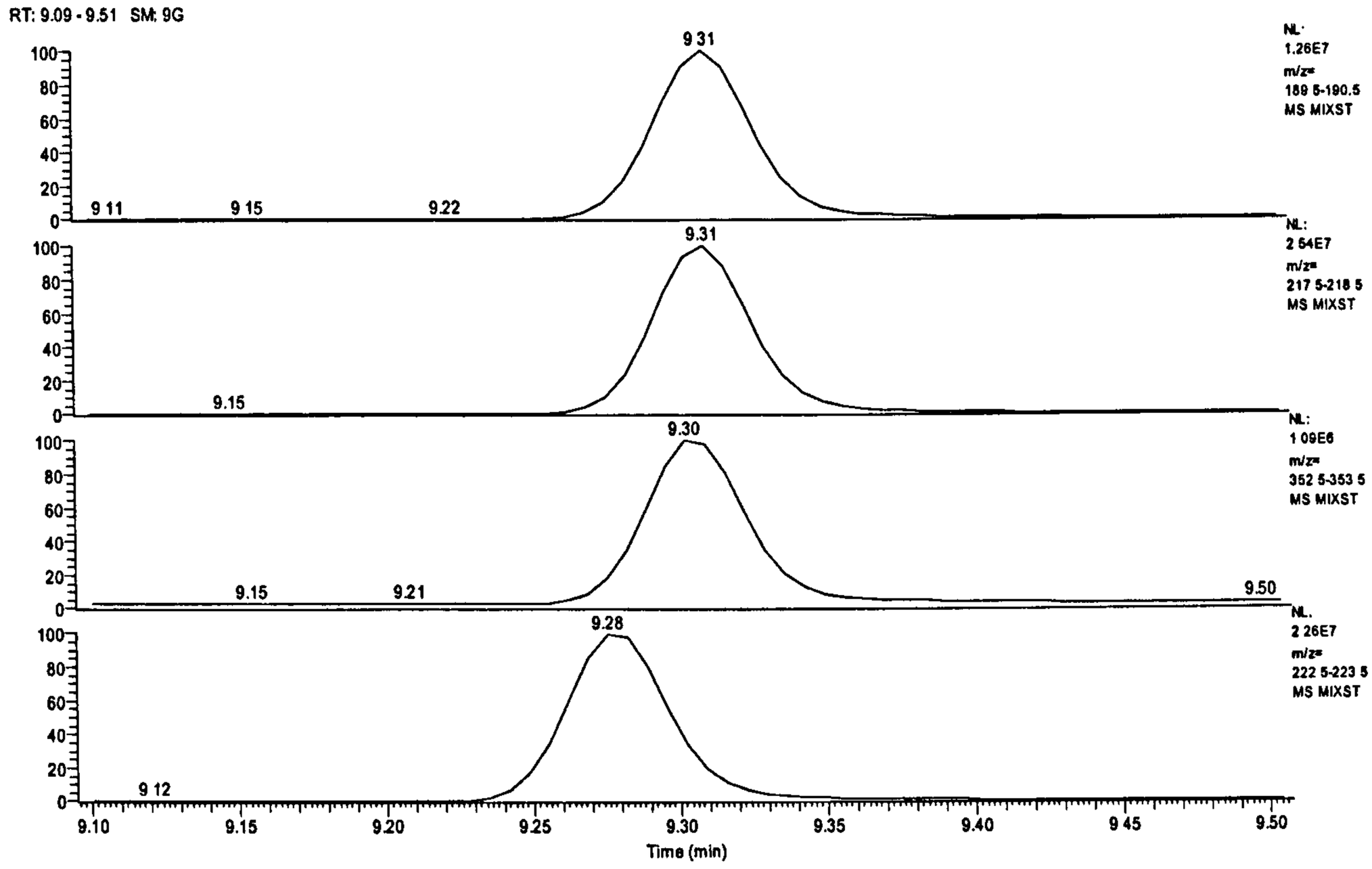


Figure 4.5 GC-MS Chromatograms of PFP derivatives of MDEA and MDEA-d_5

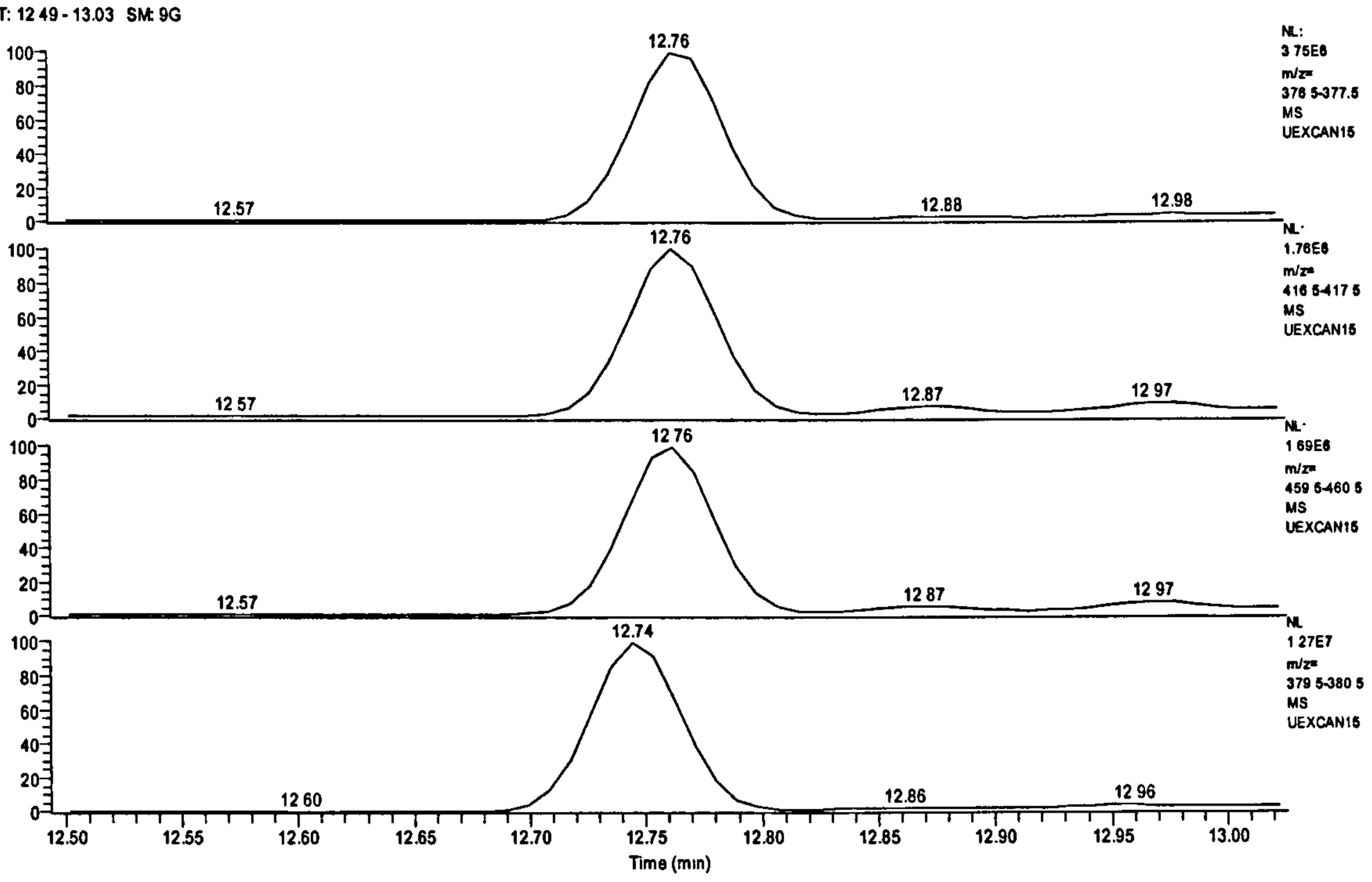


Figure 4.6 GC-MS chromatograms of PFP / PFPOH derivatives of Δ^9-THC and Δ^9-THC -d_3

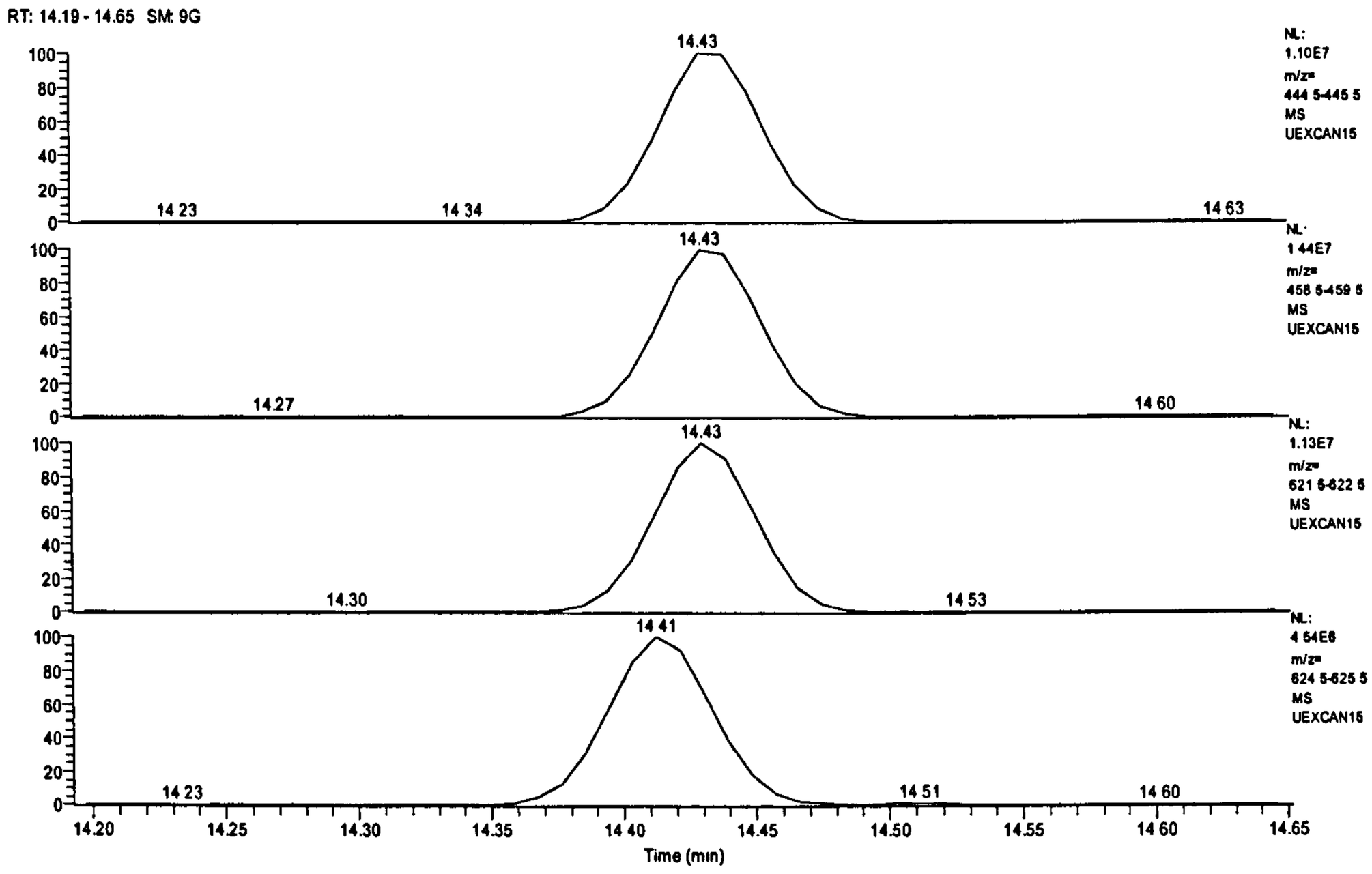


Figure 4.7 GC-MS chromatograms of PFP / PFPOH derivatives of Δ^9-THC-COOH and Δ^9-THC-COOH-d_3

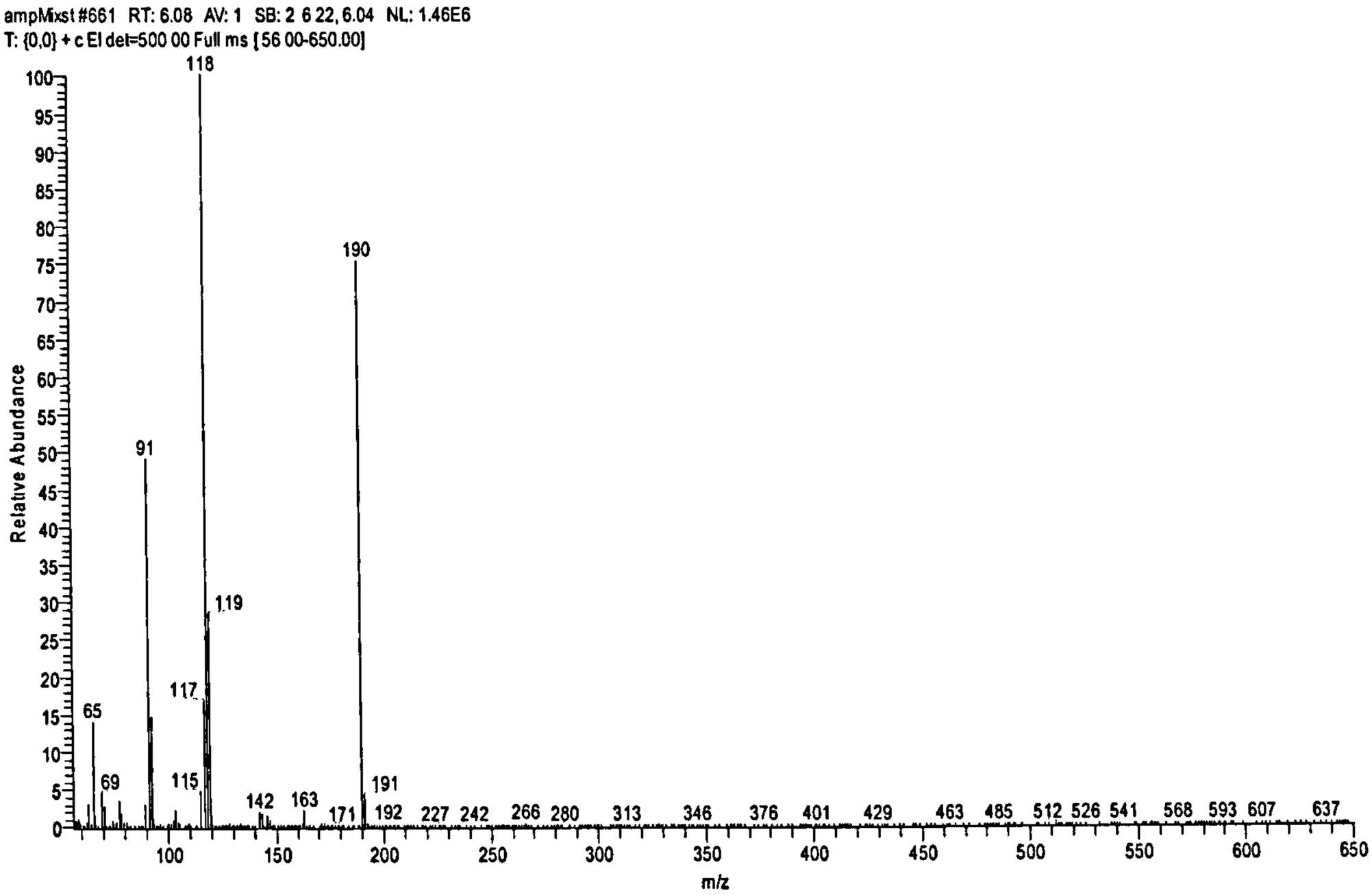


Figure 4.8 Full scan Mass Spectrum of PFP derivative of AF

Figure 4.9 Full scan Mass Spectrum of PFP derivative of MA

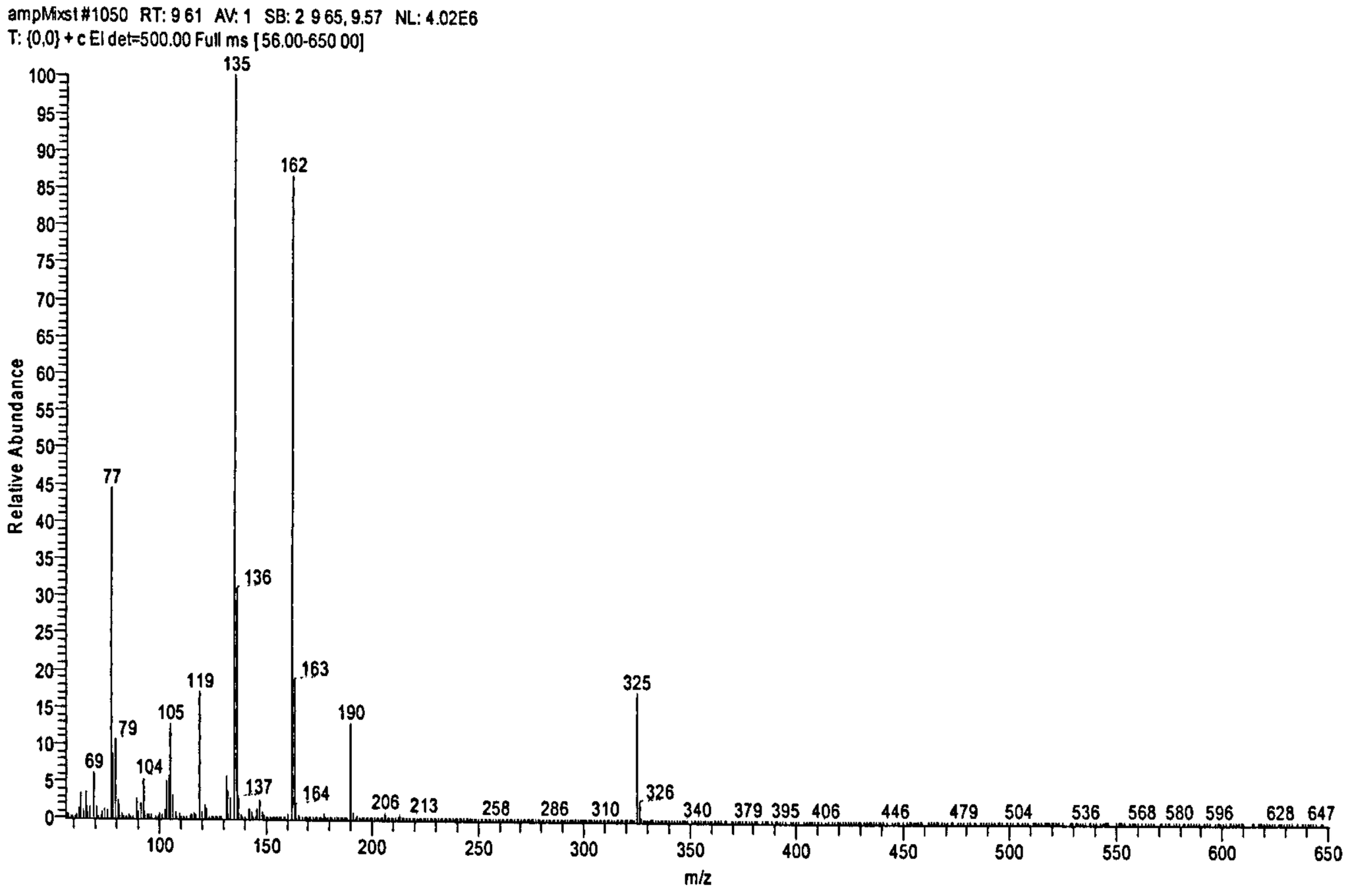


Figure 4.10 Full scan Mass Spectrum of PFP derivative of MDA

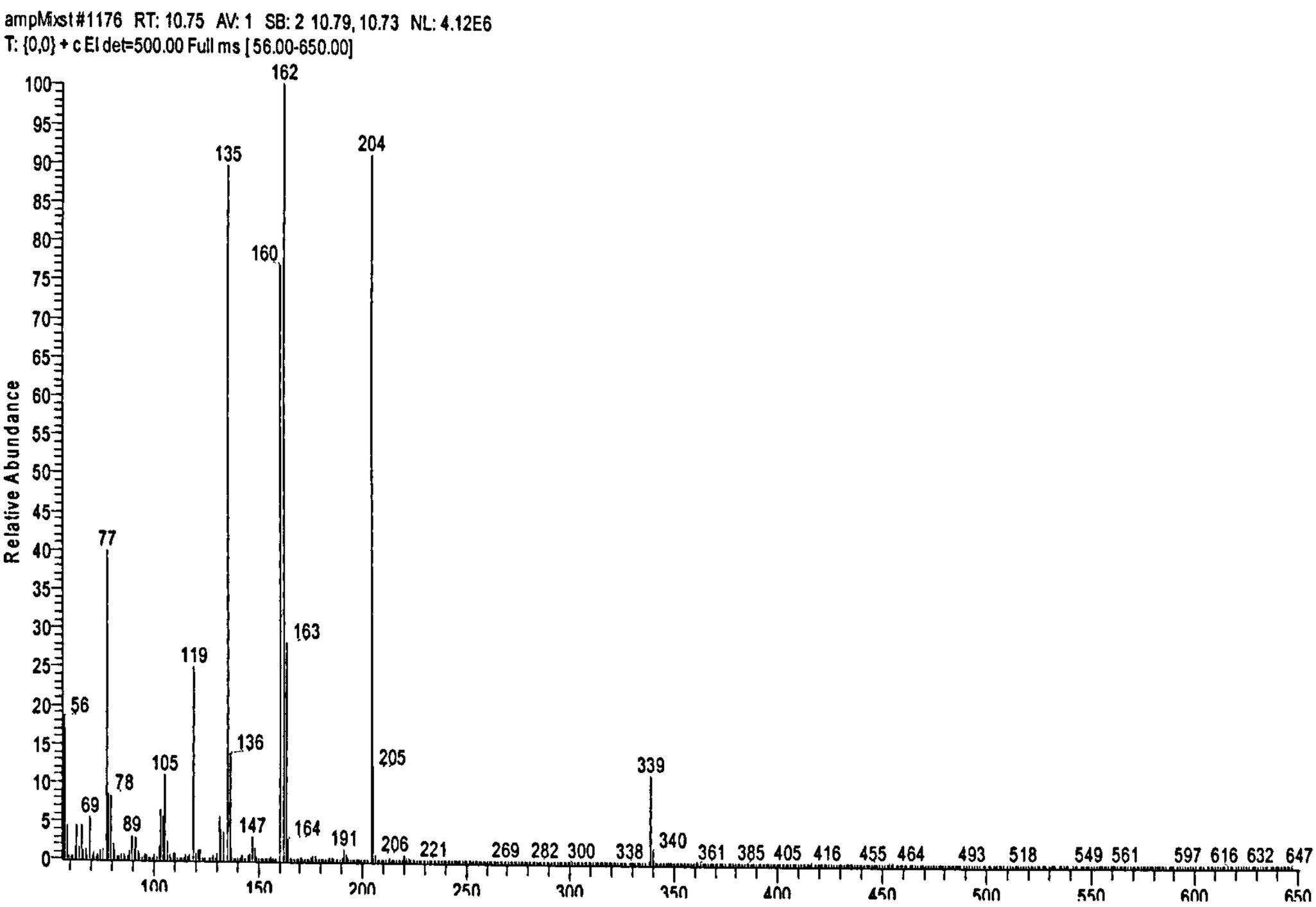


Figure 4.11 Full scan Mass Spectrum of PFP derivative of MDMA

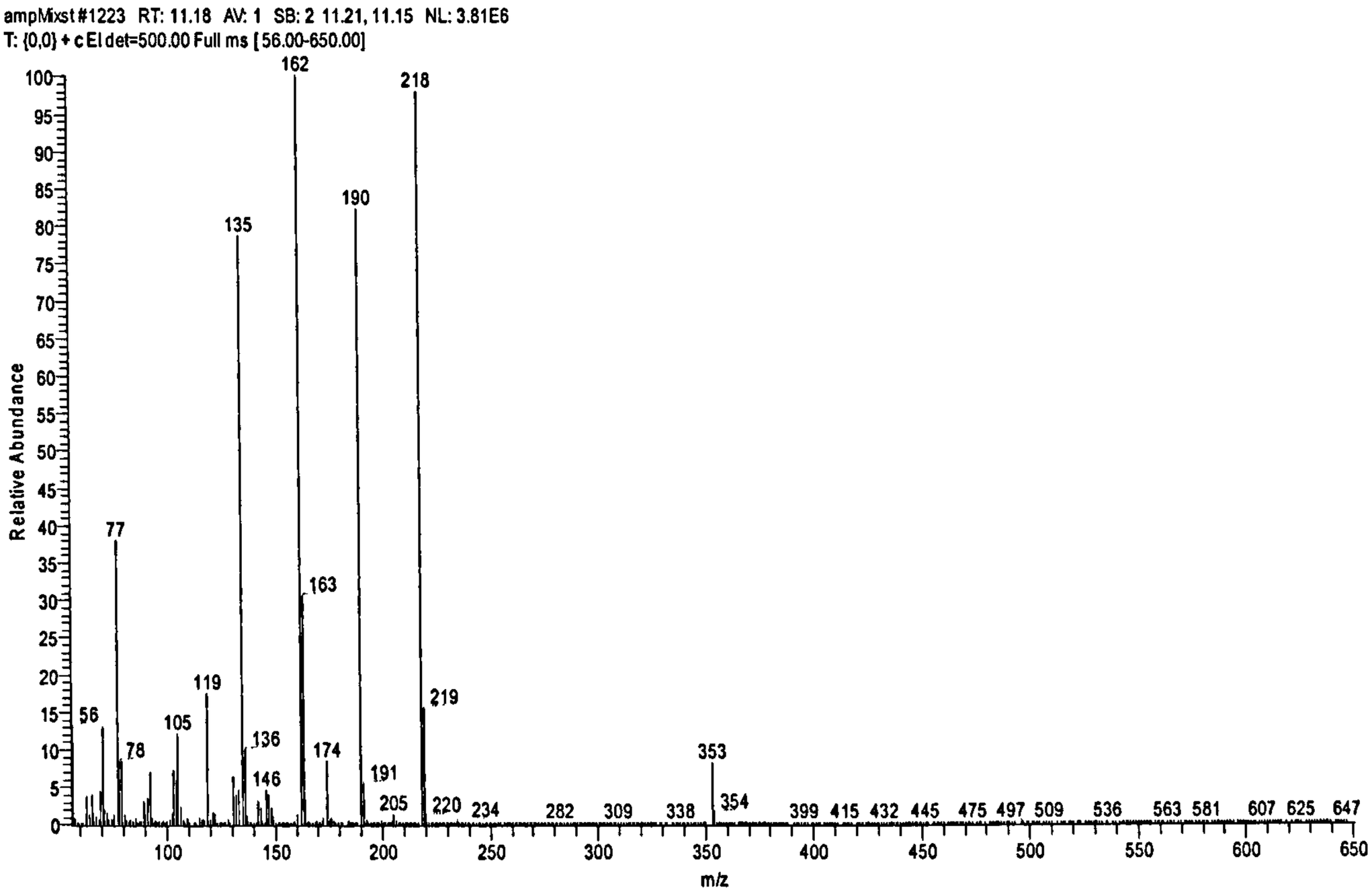


Figure 4.12 Full scan Mass Spectrum of PFP derivative of MDEA

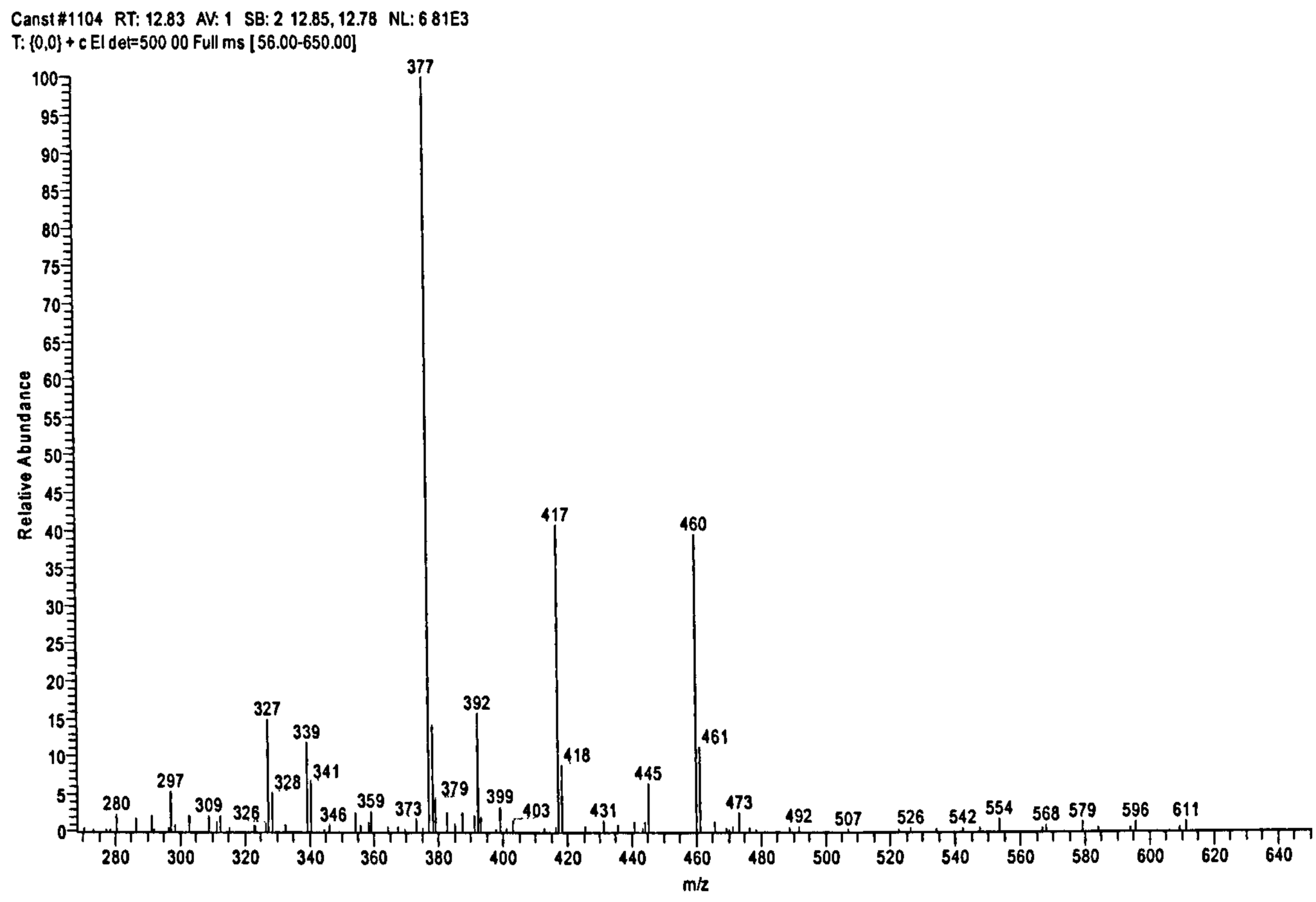


Figure 4.13 Full scan Mass Spectrum of PFP derivative of Δ^9-THC

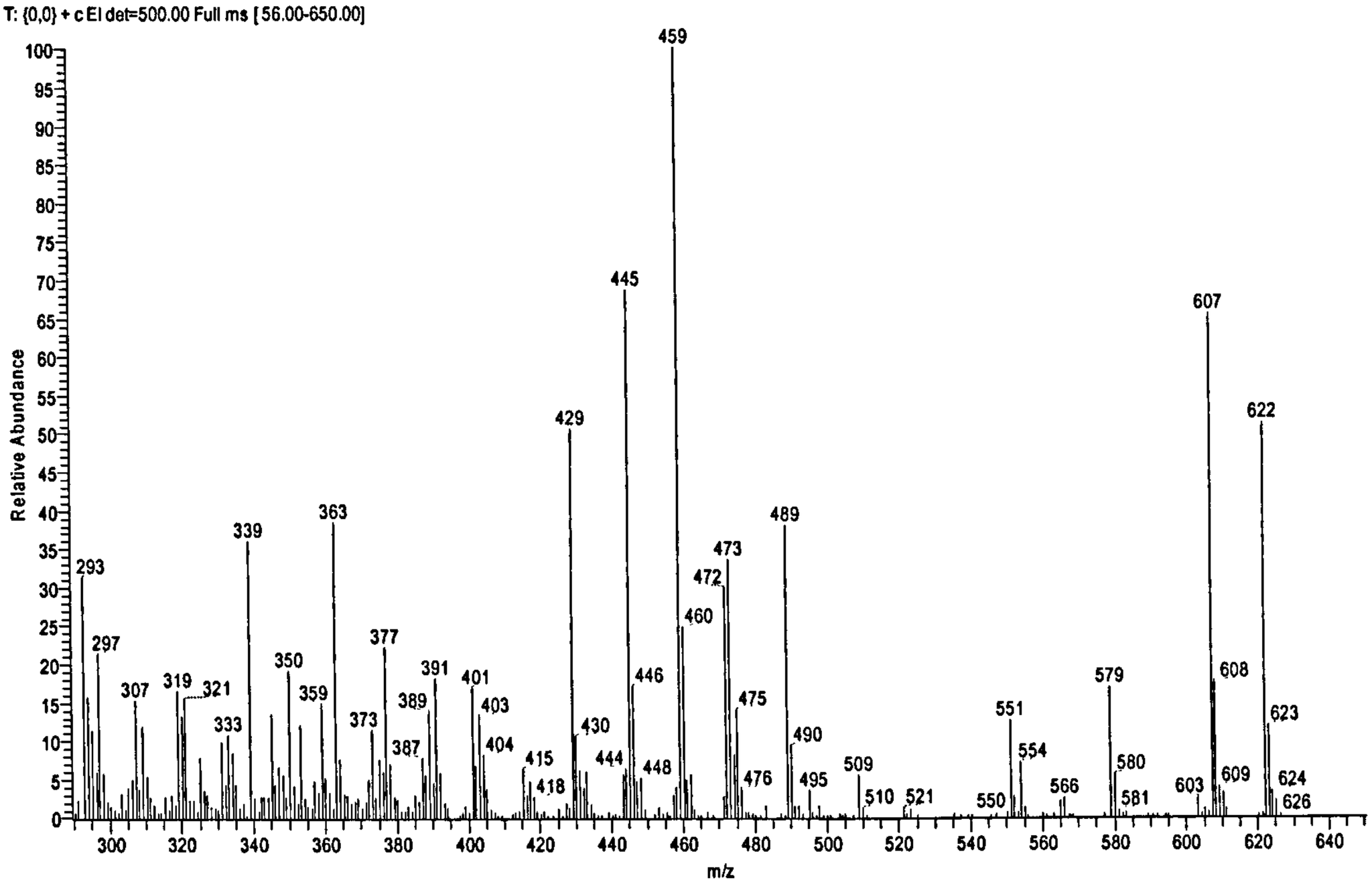


Figure 4.14 Full scan Mass Spectrum of PFP / PFPOH derivative of Δ^9-THC-COOH

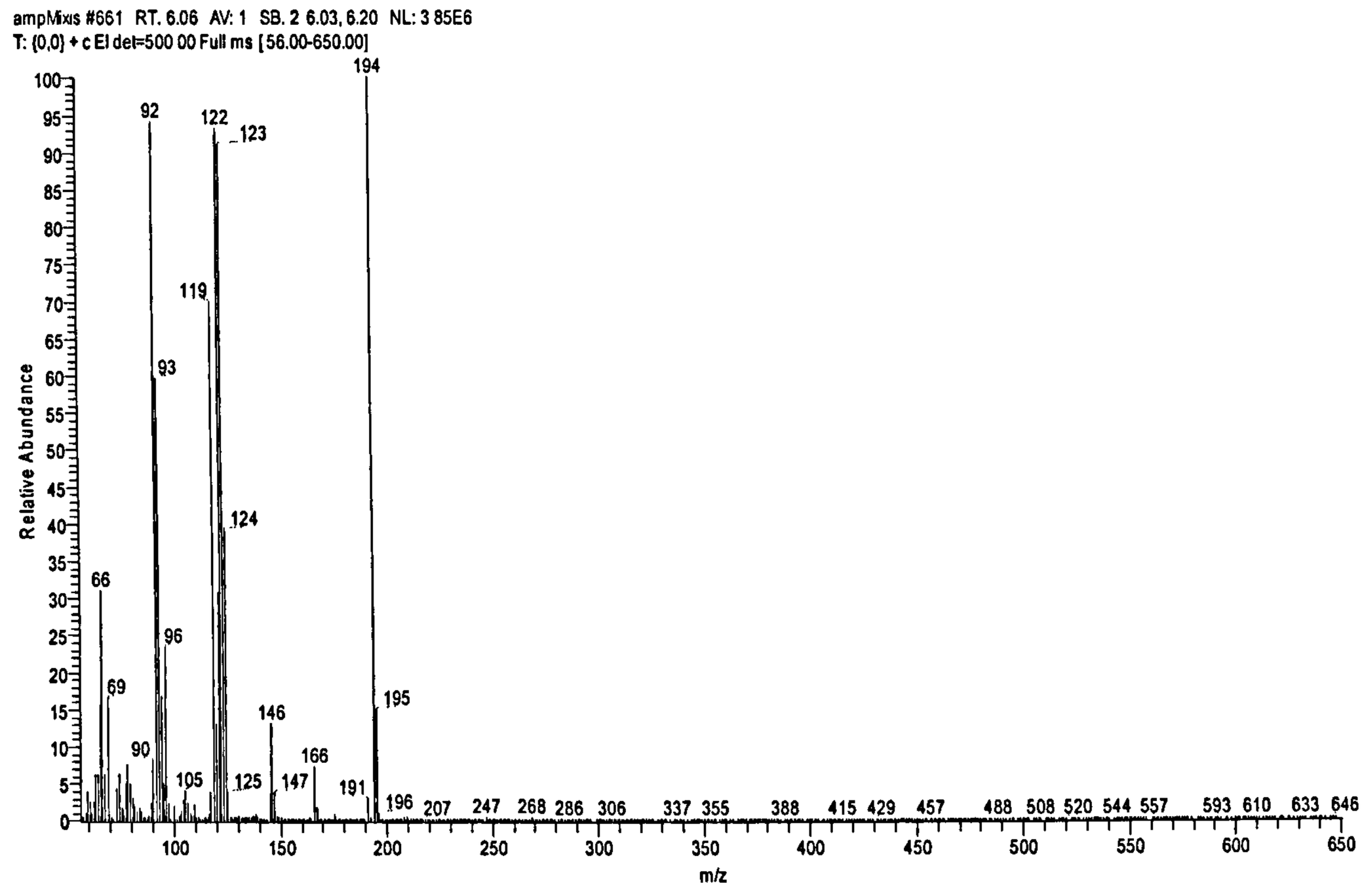


Figure 4.15 Full scan Mass Spectrum of PFP derivative of AF-d_5

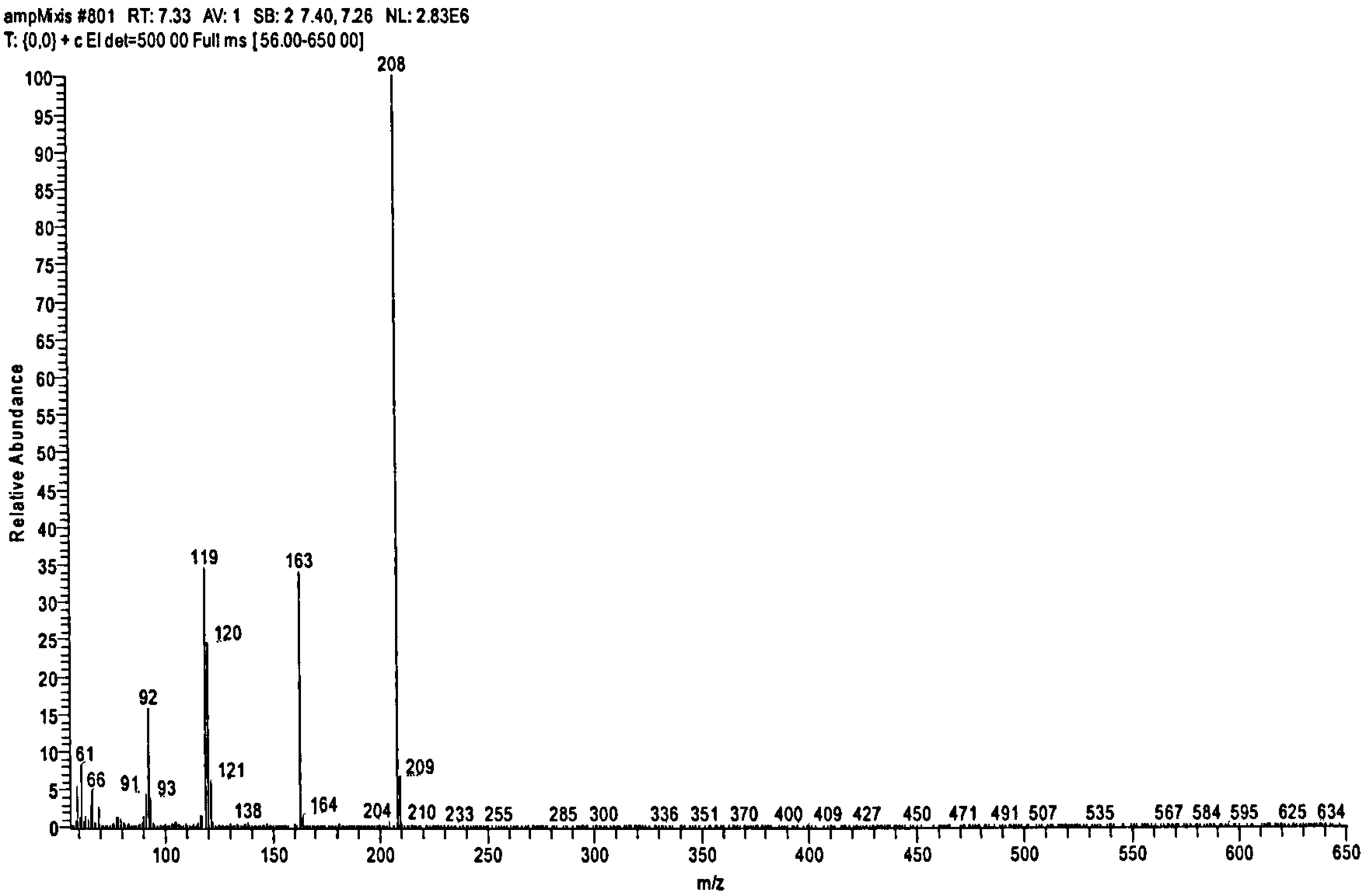


Figure 4.16 Full scan Mass Spectrum of PFP derivative of MA-d_5

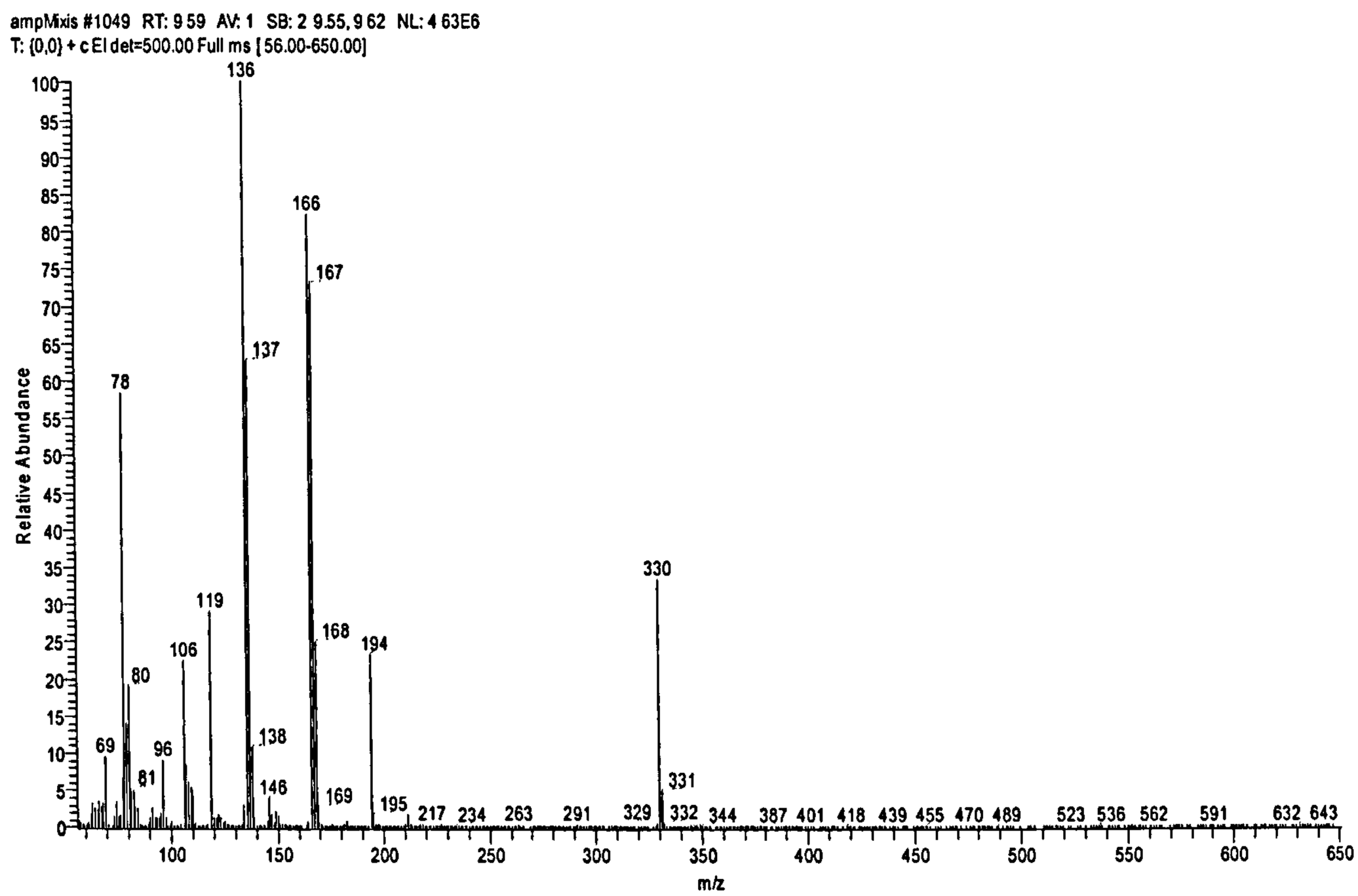


Figure 4.17 Full scan Mass Spectrum of PFP derivative of MDA-d_5

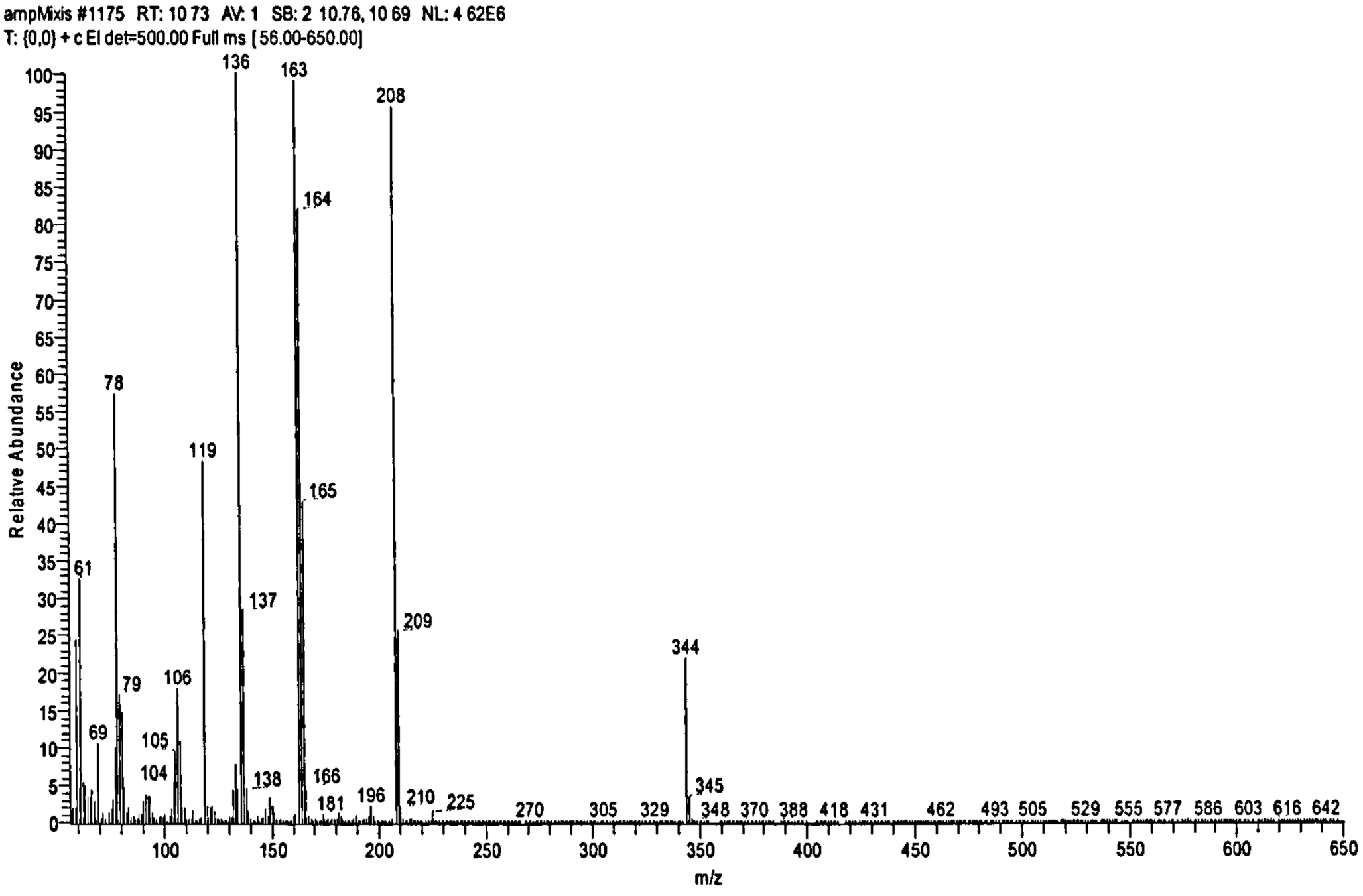


Figure 4.18 Full scan Mass Spectrum of PFP derivative of MDMA-d_5

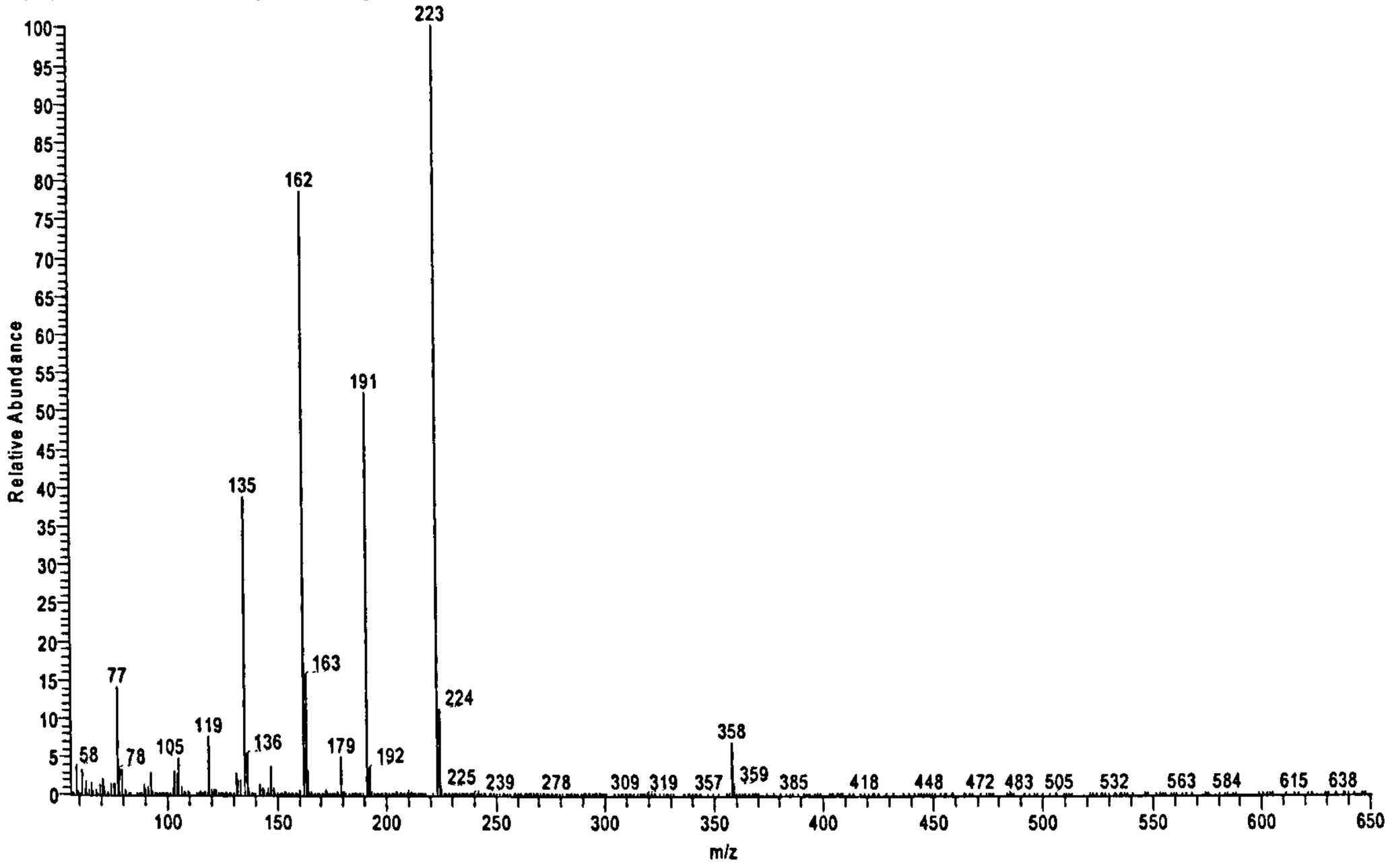


Figure 4.19 Full scan Mass Spectrum of PFP derivative of MDEA-d_5

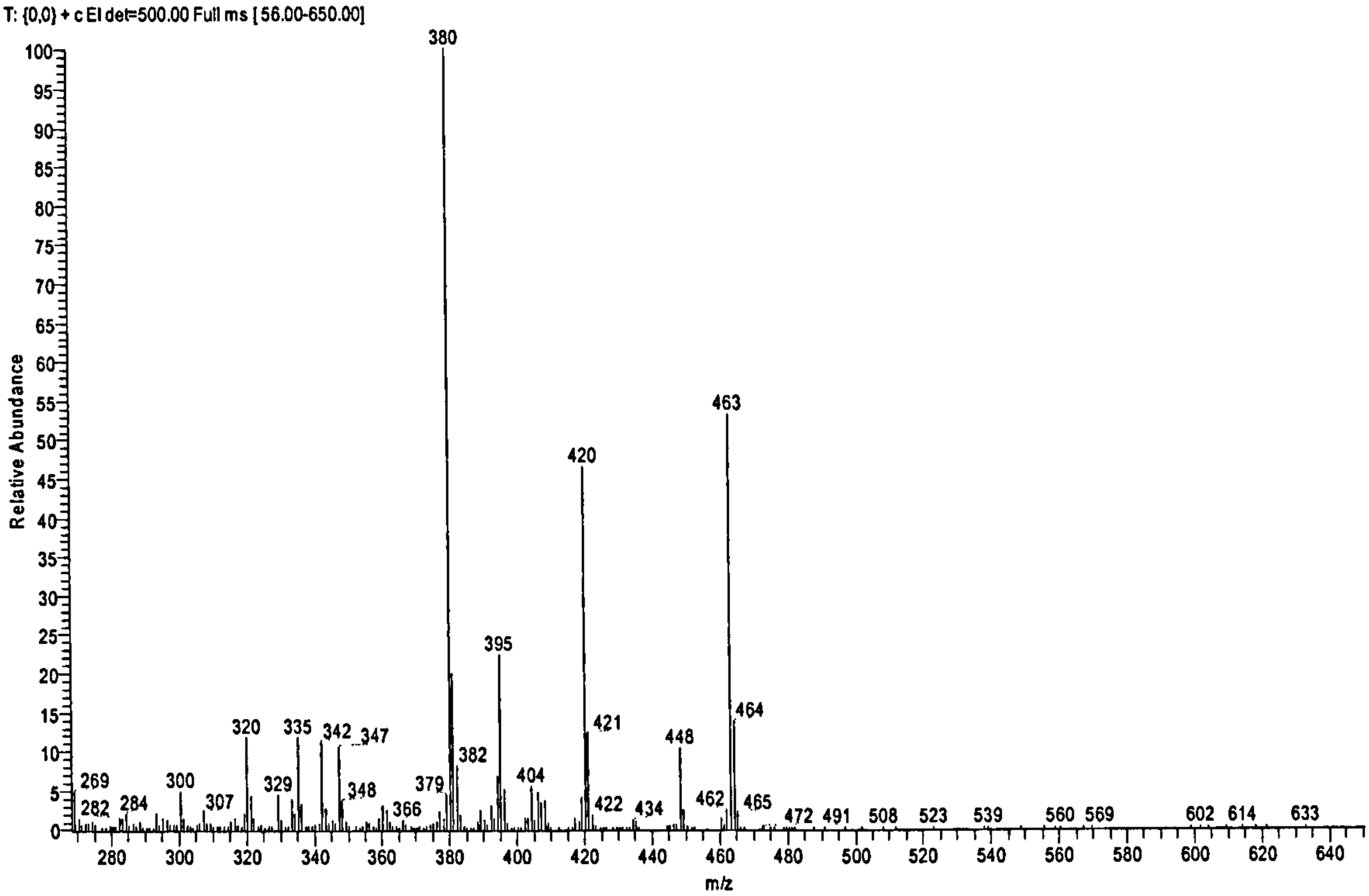


Figure 4.20 Full scan Mass Spectrum of PFP derivative of Δ^9-THC -d_3

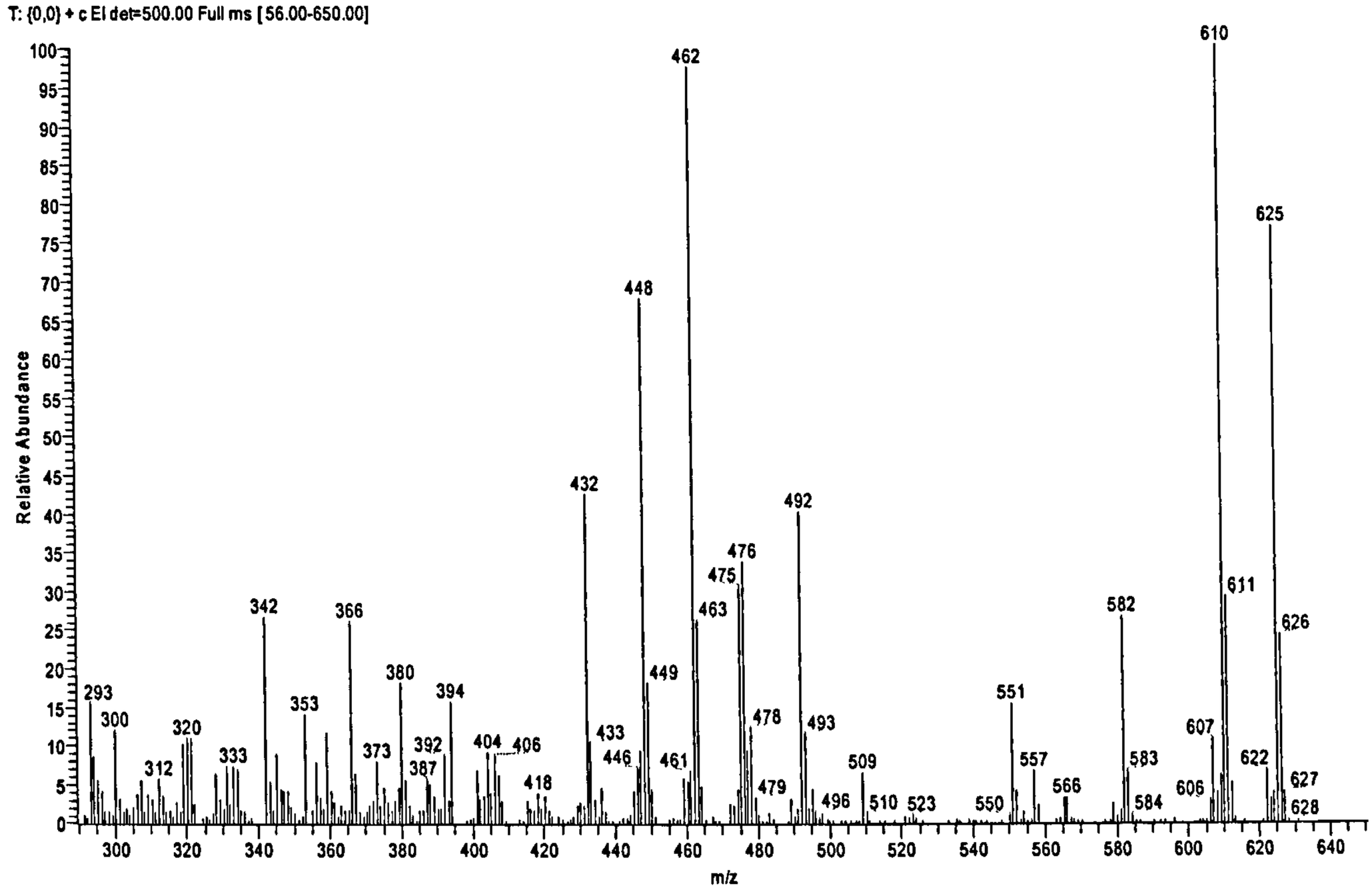


Figure 4.21 Full scan Mass Spectrum of PFP / PFPOH derivative of THC-COOH-d_3

4.1.1.5 Optimisation of Derivatisation

In order to optimise the derivatisation procedure for amfetamines and cannabinoids, 100 µl of amfetamines standard solution, cannabinoids standard solution and their deuterated standards all at 1 µg/ml were added to a vial and prepared in duplicate. The derivatisation was carried out at three different ratios of PFPA / EtOAc (2:1, 1:2, 1:1) for amfetamines and three different ratios of PFPA / PFPOH (1:1, 1:0.75, 1:2) for cannabinoids.

4.1.1.5.1 Results

All chromatograms gave good peak shape, but the use of different ratios of PFPA / EtOAc and PFPA / PFPOH gave different intensities for the drugs of interest. The quantitation ion was used to assess the intensity. The results are presented in Table 4.2. PFPA / EtOAc (2:1 v/v) gave the highest intensity for all the five amfetamines (AF, MA, MDA and MDEA) and this ratio was selected for amfetamines derivatisation.

PFPA / PFPOH (1: 0.75 v/v) was selected for the cannabinoids because the highest intensity ratio was achieved for Δ^9-THC. PFPA / PFPOH (1:1 v/v) gave the highest intensity for Δ^9-THC-COOH. However, there was little difference observed between this ratio and the one at 1:0.75. The complete results are presented in Table 4.3.

Therefore, the extracted solution by SPE was evaporated at room temperature under a slow flow of nitrogen and derivatised using 60 µl of PFPA / EtOAc (2: 1 v/v) for amfetamines and PFPA / PFPOH (1:0.75 v/v) for cannabinoids in a sealed vial which was vortexed and then placed into a 60 °C heating block for 30 minutes. After derivatisation was completed, the reagents were evaporated under nitrogen and reconstituted in 30 µl EtOAc, 1µl of which was injected for analysis by GC-MS.

Table 4.2 The mean peak area of amfetamines with different ratios of derivatising agent (PFPA / EtOAc)

Derivatising Reagent Ratio PFPA : EtOAc	Average Peak Area				
	AF	MA	MDA	MDMA	MDEA
	190 m/z	204 m/z	325 m/z	339 m/z	218 m/z
2:1	2090826	5102208	776362	357643	10384886
1:2	1554504	3993772	460207	224711	3763808
1:1	1524217	1296367	714397	329090	6012077

Table 4.3 The mean peak area of cannabinoids with different ratios of derivatising agents (PFPA / PFPOH)

Derivatising Reagent Ratio PFPA : PFPOH	Average of Peak Area Ratios	
	Δ^9-THC	Δ^9-THC-COOH
	377 m/z	622 m/z
1:1	2768566	43103
1: 0.75	3349724	42565
1: 2	1284828	18808

4.1.1.6 Buffers and Solutions

0.1 M phosphate buffer, pH 6.0

6.81 g of potassium dihydrogen phosphate (KH_2PO_4, FW =136.09) was weighed into a 0.5 litre volumetric flask and approximately 450 ml of distilled water was added. The pH was adjusted to 6.0 (+/ - 0.1) with 10.0 M potassium hydroxide solution while stirring and the solution was made up to 0.5 litre with distilled water and was stored at 5 °C. The solution was discarded after one month.

0.01 M acetic acid, pH 3.3

57.2 µl of glacial acetic acid was pipetted into a 100 ml volumetric flask and DI H_2O was added up to the 100 ml mark. This was mixed well and stored at room temperature. The solution was discarded after two months.

Acetone / Chloroform (1:1 v/v)

50 ml of acetone and 50 ml chloroform were measured into a bottle, mixed well and sonicated for 10 minutes, stored at room temperature and discarded after one month.

EtOAc / concentrated ammonia (98:2)

98 ml EtOAc was measured into a bottle and 2 ml of concentrated ammonia solution (ammonium hydroxide) was added carefully. This was prepared under a fume hood and sonicated for 10 minutes. This solution was made up fresh every day.

Tartaric Acid in EtOAc (1 mg/ml)

5 milligrams of tartaric acid were weighed into a large vial and 5 ml of EtOAc was added. This was sonicated for 10 minutes, stored at room temperature and discarded after one month.

1M sodium hydroxide (NaOH)

4.0 grams of NaOH (MW 40.0) pellets were measured into a 100 ml volumetric flask and made up to the 100 ml mark with DI H_2O. This was mixed thoroughly, stored at room temperature and discarded after six months.

0.1 M hydrochoric acid (HCl)

4.2 ml concentrated HCl was added to 400 ml of DI H_2O and diluted to 500 ml with DI H_2O and mixed thoroughly. This was stored at room temperature and discarded after six months.

n-hexane / EtOAc (9:1 v/v)

90 ml hexane was measured into a bottle and added 10 ml EtOAc and mixed well, stored at room temperature and discard after one month.

n-hexane / EtOAc (8:2 v/v)

80 ml hexane *was* measured into a bottle and to this 20 ml EtOAc was added and mixed well. This was stored at room temperature and discarded after one month.

4.1.1.7 Preparation and Decontamination of Hair

Drug free hair was obtained from volunteers and tested by the method, which has been used by Department of Forensic Medicine and Science, University of Glasgow, to show it was negative for amfetamines and cannabinoids. In this study, the decontamination procedure chosen was one that had been used routinely for hair analysis by Forensic Medicine & Science, University of Glasgow. Blank hair and case hair samples were decontaminated using the following wash steps:

- Immersion in 0.1 % SDS detergent, sonication for 15 minutes. Solution discarded (x 1)
- Immersion in DI H_2O, sonication for 15 minutes. Solution discarded. (x 2)
- Immersion in dichloromethane, sonication for 10 minutes. Solution collected for analysis (x 3)

If the last wash fraction of a case hair sample was positive for any of the drugs the dichloromethane wash step was repeated until the wash fraction was free of drugs. Once the last wash with dichloromethane had tested negative for the analytes of interest or was less than its limit of detection, the hair samples (both blank and case samples) were allowed to air dry overnight and were then weighed. Washing takes place prior to cutting the hair. After drying the hair overnight at room temperature, the hair samples were cut into small segments (1-2 mm) and an appropriate amount weighed out into 10-ml screw-capped tubes.

4.1.1.8 Hair Pretreatment Methods

To liberate the drug from the hair matrix, the following pre- treatment methods were used:

Alkaline pre-treatment: Hair was incubated in 1 ml of 1M NaOH at 95 °C for 10 minutes. After cooling to room temperature, 1 ml of 1 M HCl was added to neutralize the solution and 2 ml of phosphate buffer, pH 6.0 was added. After adjusting with 1 M NaOH to pH 6.0 and centrifuging for 20 minutes at 2000 rpm, the supernatant was transferred to a clean large vial for extraction.

β-Glucuronidase pre-treatment (helix pamatia, 86,900 unit / ml): Hair was incubated in 1ml of phosphate buffer pH 6 and 50 µl β-glucuronidase solution at 45 °C for 2 hours. After cooling to room temperature, 2 ml of phosphate buffer pH 6.0 was added, vortex

mixed and centrifuged for 20 minutes at 2000 rpm. The supernatant was transferred to a clean large vial for extraction.

Methanol pre-treatment: Hair was incubated in 1 ml of (MeOH) at 45 °C for 18 hours. After cooling to room temperature, the MeOH was transferred into a clean tube, 4 ml of phosphate buffer pH 6.0 was added, vortex mixed and centrifuged for 20 minutes at 2000 rpm. The supernatant was transferred to a clean large vial for extraction.

Acid pre-treatment: Hair was incubated in 1ml of dilute acid (0.1 HCl) at 45 °C for 12 hours. After cooling to room temperature, 1 ml of 0.1 M NaOH was added to neutralize the solution and 2 ml of phosphate buffer pH 6.0 was added. This was centrifuged for 20 minutes at 2000 rpm and the supernatant was transferred to a clean large vial for extraction.

4.1.1.9 Solid Phase Extraction Method for Amfetamines and Cannabinoids

The SPE method developed by *Chen et al* was chosen as the clean-up method because it has been used to isolate a broad range of drugs (acidic, neutral and basic drugs) from whole blood with acceptable recoveries. Most drugs (acidic, neutral and basic) are retained on the column at pH 6 [159]. This method was investigated as a possible clean-up step for the combined extraction of amfetamines and cannabinoids in hair.

SPE was carried out using Bond Elut Certify™ columns. The SPE columns were conditioned with 2 ml of methanol and 2 ml of phosphate buffer (pH 6, 0.1M) in preparation to receiving an aqueous sample. The samples were applied to the column, taking at least two minutes to pass through the column. The column was then washed with 1 ml DI H_2O to remove endogenous and interference components. 0.5 ml 0.01 M acetic acid (pH 3.3) was added to adjust the pH of extraction. It was reported that pH 3.3 was the best choice to adjust the extraction system to elute the acids or basic drugs selectively from the column. The column was dried under full vacuum for 5-10 minutes and 50 µl of methanol was added. This was dried for a further 3 minutes under full vacuum. The cannabinoids were eluted with 3.5 ml of acetone/chloroform (1:1 v/v) [162]. The amfetamines were eluted with 2 ml of 2% concentrated ammonia in ethyl acetate [159, 162]. 100 µl tartaric acid was added to prevent amfetamines from evaporating [114]. The elution solutions were evaporated at room temperature under a slow flow of nitrogen and the acetone / chloroform fraction residue was derivatised as described in section 4.1.1.5.1

for cannabinoids and the EtOAc / ammonia fraction residue was derivatised as described in section 4.1.1.5.1 for amfetamines.

4.1.1.10 Calibration Curves

The purpose of this initial experiment was to investigate the simultaneous extraction of amfetamines and cannabinoids using the four pre-treatment-methods. Each of the pre-treatment methods described in section 4.1.1.8 were used on 30 mg hair samples which were spiked with 5, 10, 25, 50, 100 and 200 µl AF, MA, MDA, MDMA, MDEA, Δ^9-THC and Δ^9 -THC-COOH of working standard solution and 100 µl of deuterated working standard, all at 1 µg/ml to give a six point calibration curve. A blank hair sample without standard or internal standard and a blank hair sample with only 100 µl of internal standard were also prepared and extracted. The solid phase method described in 4.1.1.9 was used to clean up the hair samples. These were derivatised as described in sections 4.1.1.5.1.

4.1.1.10.1 Calibration Curves Result

The peak area ratios (PAR) of AF, MA, MDA, MDMA and MDEA to their deuterated internal standards were calculated and calibration curves of PAR against concentration were plotted for each compound. The results demonstrated that all extraction methods worked well for amfetamines which had been added to hair. The linearities are summarised in Table 4.4 and Table 4.5, and shown in Figure 4.22, Figure 4.23, Figure 4.24, Figure 4.25 and Figure 4.26 .

In contrast, the cannabinoid results were poor. The detection limits for these appeared to be low as shown by their low peak areas in comparison to unextracted standards. Calibration curves could not be plotted for these and further investigation was required to improve the recovery.

Table 4.4 Linearity of amfetamines with different extraction methods

Analytes	Concentration	Correlation coefficients (R^2)			
	Range ng/30mg	Enzymatic	Alkaline	Acid	Methanol
AF	5 → 200	1.000	0.998	0.994	0.998
MA	5 → 200	1.000	0.997	0.994	1.000
MDA	5 → 200	0.999	0.994	0.999	0.992
MDMA	5 → 200	0.999	0.996	0.999	0.999
MDEA	5 → 200	1.000	0.997	1.000	1.000

Table 4.5 Linear Values of amfetamines with different extraction methods

Analyte	Enzymatic		Alkaline		Acid		Methanol	
	Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope
AF	0.0267	0.0121	-0.0100	0.0097	0.0087	0.0092	-0.0494	0.0082
MA	0.0109	0.0074	-0.0159	0.0097	0.0360	0.0071	-0.0382	0.0125
MDA	0.0360	0.0121	-0.0524	0.0107	-0.0379	0.0120	-0.1477	0.0145
MDMA	0.0164	0.0124	0.0124	0.0103	-0.0517	0.0120	-0.0037	0.0113
MDEA	0.0037	0.0017	-0.0295	0.0118	-0.0219	0.0095	-0.0067	0.0080

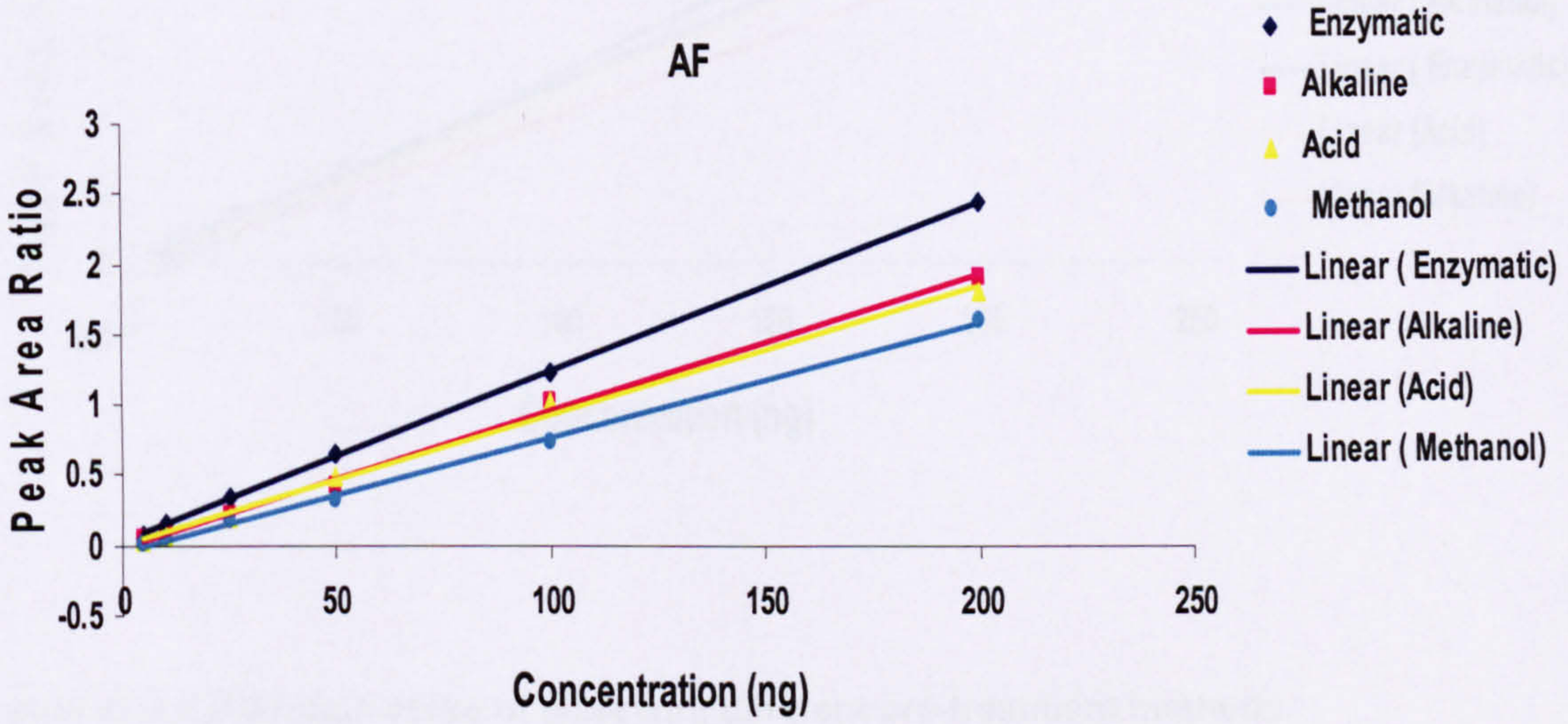


Figure 4.22 Calibration curve of AF with different pre-treatment methods

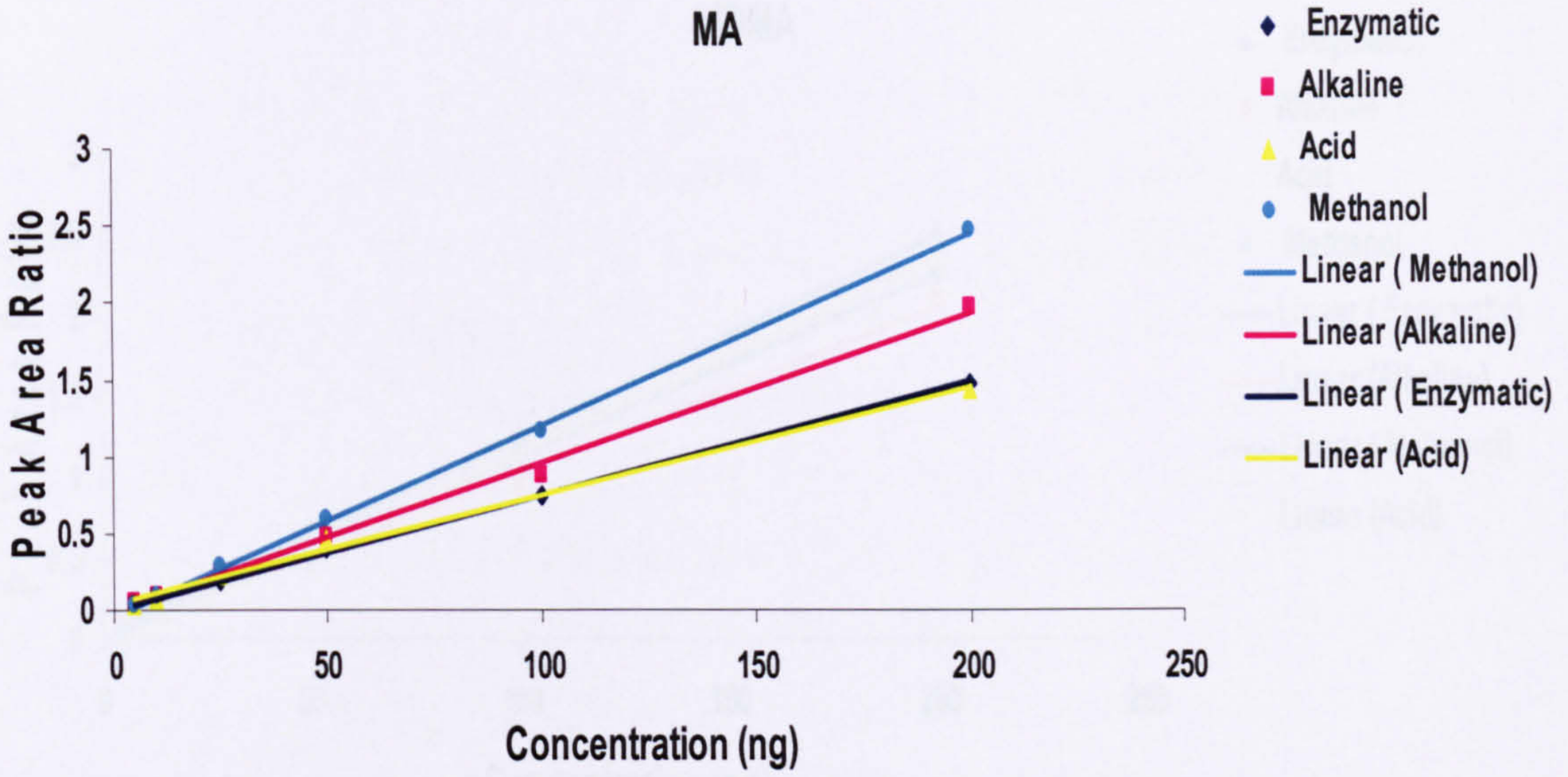


Figure 4.23 Calibration curve of MA with different pre-treatment methods

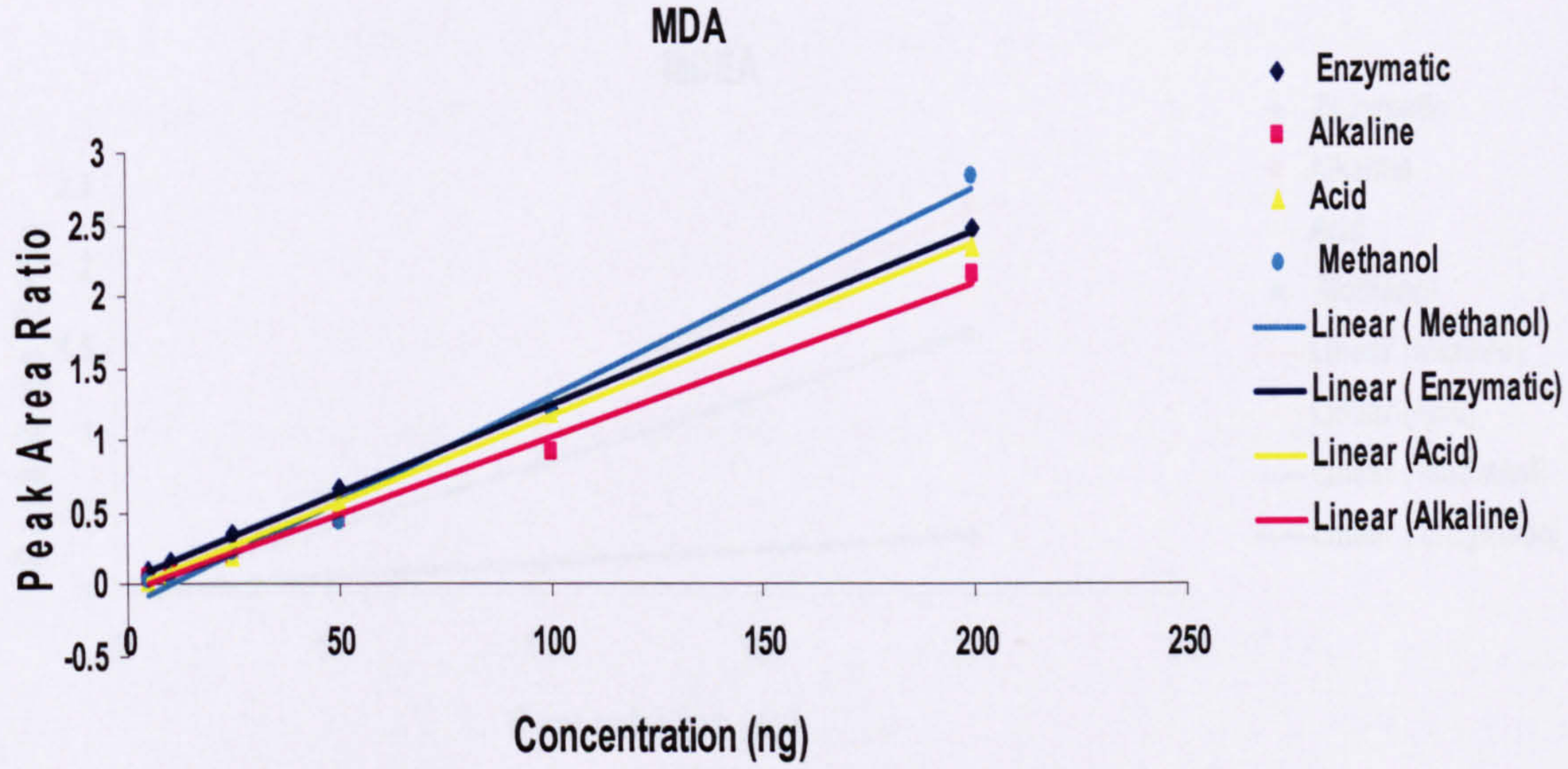


Figure 4.24 Calibration curve of MDA with different pre-treatment methods

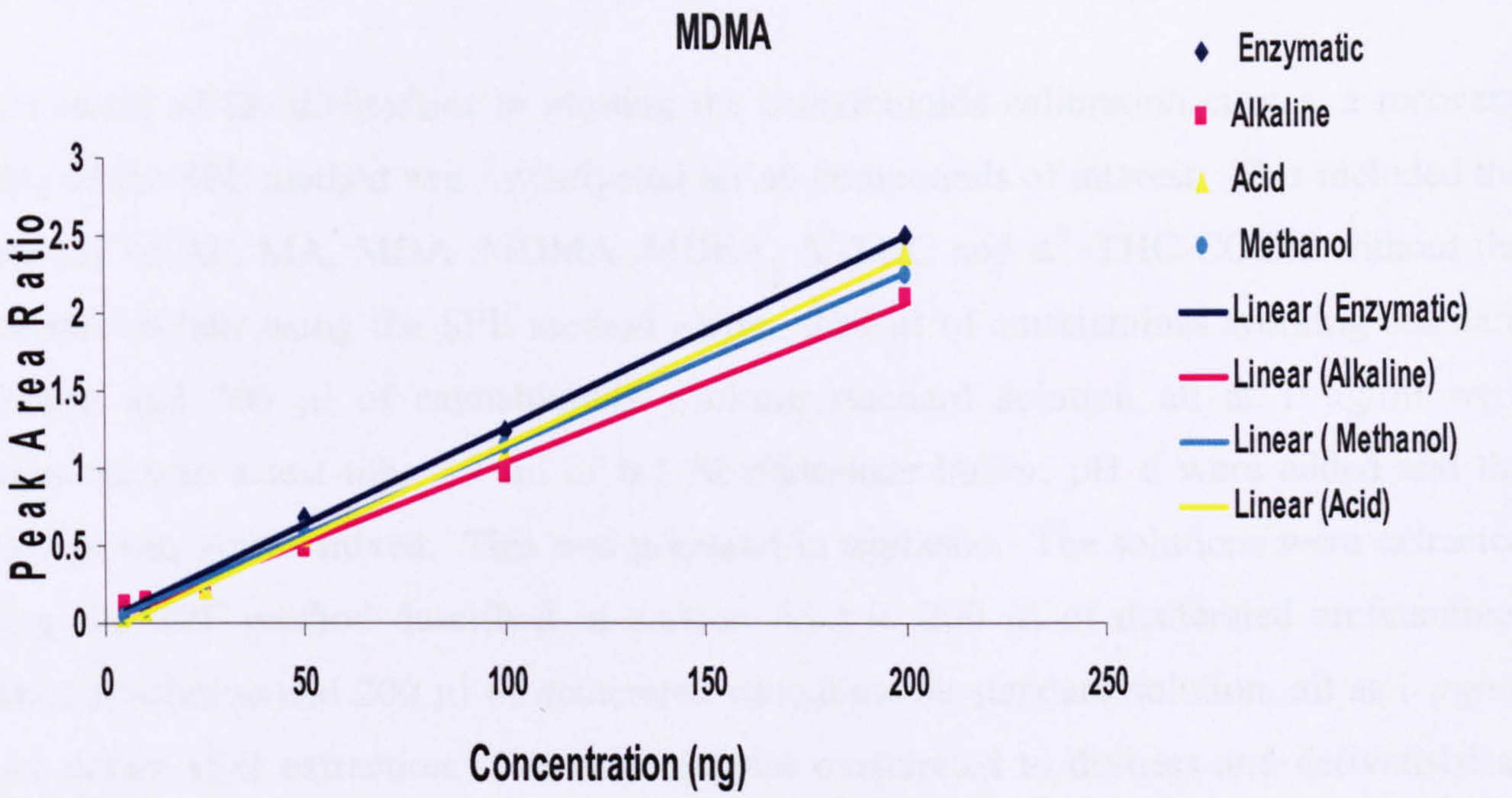


Figure 4.25 Calibration curve of MDMA with different pre-treatment methods

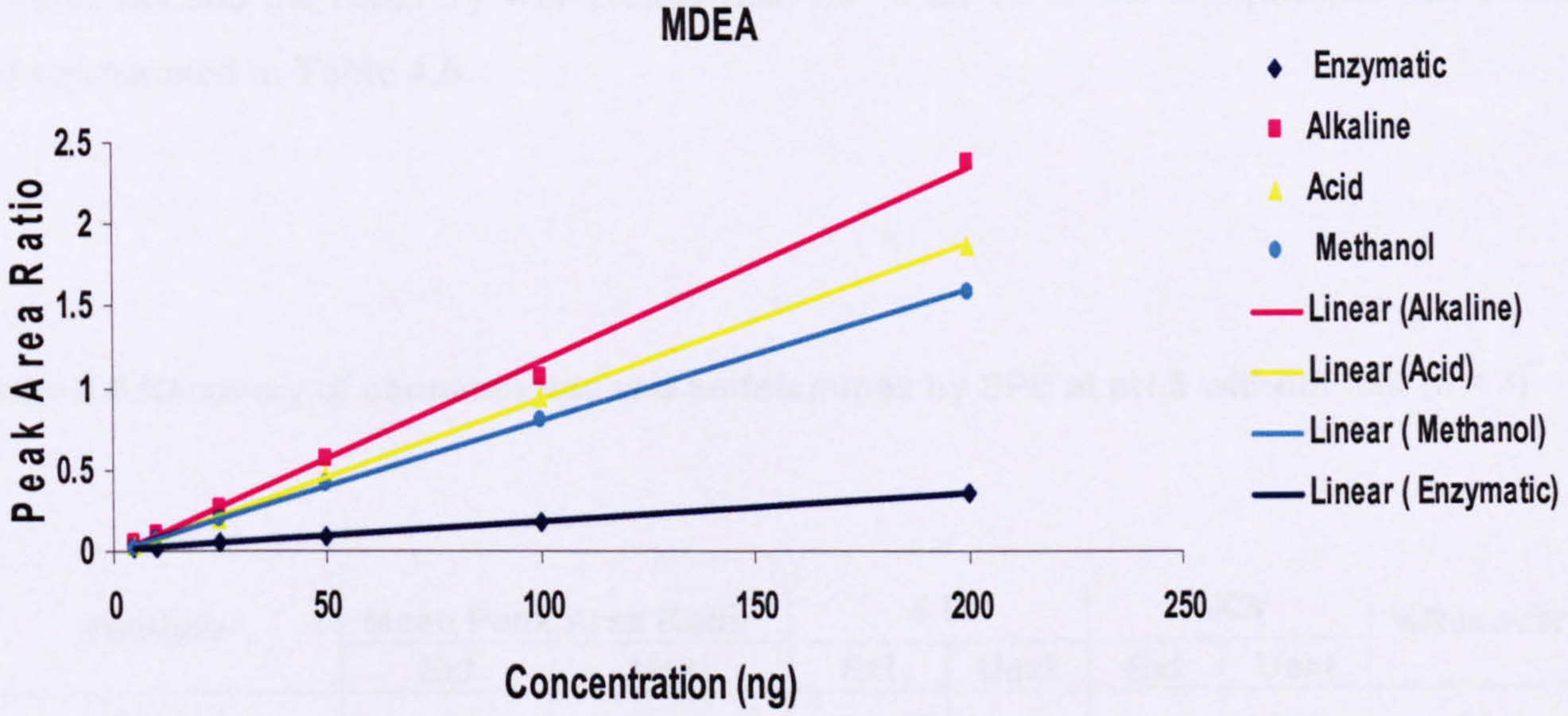


Figure 4.26 Calibration curve of MDEA with different pre-treatment methods

4.1.1.11 SPE Recovery Study

As a result of the difficulties in plotting the cannabinoids calibration curves, a recovery study of the SPE method was investigated for all compounds of interest. This included the recovery of AF, MA, MDA, MDMA, MDEA, Δ^9-THC and Δ^9 -THC-COOH without the presence of hair using the SPE method alone. 200 µl of amfetamines working standard solution and 200 µl of cannabinoids working standard solution all at 1 µg/ml were measured into a test tube. 3 ml of 0.1 M phosphate buffer, pH 6 were added and the solution was vortex mixed. This was prepared in triplicate. The solutions were extracted using the SPE method described in section 4.1.1.9. 200 µl of deuterated amfetamines standard solution and 200 µl of deuterated cannabinoids standard solution, all at 1 µg/ml were added after extraction. The solution was evaporated to dryness and derivatised as described in section 4.1.1.5.1.

4.1.1.11.1 Recovery Study Results

The recoveries were determined by comparing the PAR of extracted standards to the PAR of the unextracted standards. The results showed that the SPE was successful for all of the compounds and the recovery was greater than 64 % for all of the compounds. The results are represented in Table 4.6.

Table 4.6 Recovery of cannabinoids and amfetamines by SPE at pH 6 without hair (n = 3)

Analyte	Mean Peak Area Ratio		S.D		%CV		%Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Δ^9- THC	0.10	0.16	0.01	0.01	7.9	3.4	64.2
Δ^9 -THC-COOH	1.11	1.54	0.23	0.06	20.4	3.8	72.1
AF	1.31	1.73	0.07	0.35	5.5	20.1	75.4
MA	0.93	1.20	0.11	0.05	11.6	4.2	77.6
MDA	0.96	1.17	0.12	0.00	12.8	0.4	82.2
MDMA	1.35	1.65	0.20	0.01	14.9	0.3	81.5
MDEA	0.13	0.15	0.01	0.00	5.6	2.2	89.0

Ext: Extracted, Uext: Unextracted

4.1.1.12 Recovery Study Using Alkaline Digestion Followed by SPE

The recoveries of AF, MA, MDA, MDMA and MDEA, Δ^9-THC and Δ^9-THC-COOH from spiked hair using alkaline pre-treatment followed by SPE were investigated. Alkaline pre-treatment was chosen initially because of its ability to completely dissolve the hair matrices and therefore to release the drugs completely from authentic hair samples [163]. 200 µl of amfetamines working standard solution and 200 µl of cannabinoids working standard solution all at 1 µg/ml were measured into a test tube containing 30 mg of blank hair. 1 ml of 1M NaOH was added and the solution was vortex mixed. The solution was incubated at 95 °C for 10 minutes. After cooling to room temperature, 1 ml of 1 M HCL was added to neutralize the solution and 2 ml of phosphate buffer, pH 6.0 was added. After adjusting to pH 6.0 with 1.0 M NaOH, the solution was centrifuged for 20 minutes at 2000 rpm and the supernatant transferred to a clean large vial for extraction as described in section 4.1.1.9. This was prepared in triplicate. The deuterated working standards were added after the extraction. The solution was evaporated to dryness and derivatised as described in section 4.1.1.5.1.

4.1.1.12.1 Recovery Results

The recoveries were determined by comparing the PAR of extracted spiked blank hair (30 mg) standards to the PAR of the unextracted standards. The results showed that the recovery of cannabinoids had significantly decreased when alkaline pre-treatment was used followed by SPE. On the other hand the amfetamines were not affected. The results are represented in Table 4.7.

Table 4.7 Recovery of cannabinoids and amfetamines by alkaline pre-treatment (n = 3)

Analyte	Mean Peak Area Ratio		S.D		%CV		%Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Δ^9- THC	0.21	1.50	0.04	0.11	18.3	7.1	13.8
Δ^9 -THC-COOH	0.24	2.04	0.04	0.35	18.3	17.4	11.9
AF	1.42	1.77	0.05	0.03	3.8	1.8	80.2
MA	0.58	0.66	0.05	0.03	8.0	4.8	88.7
MDA	0.75	0.90	0.01	0.03	0.7	3.4	82.8
MDMA	0.89	1.14	0.02	0.05	2.7	4.2	77.9
MDEA	0.11	0.13	0.00	0.01	1.3	4.3	80.7

Ext: Extracted, Uext: Unextracted

4.1.1.13 Conclusion

The low recoveries obtained for cannabinoids after alkaline pre-treatment followed by SPE showed that the method was not sufficiently sensitive for the detection and quantification of cannabinoids in hair. Consequently, the method required further investigation to improve the recovery of the cannabinoids by this proposed method. Also, the inability to plot calibration curves for all pretreatment methods showed that further investigation was needed.

4.1.1.14 Method Modification for Cannabinoids

4.1.1.14.1 Change in Eluent

Further investigations were carried out with a minor modification of the method by using four different eluents for the cannabinoids. These were acetone/chloroform (1:1 v/v), hexane / EtOAc (9:1 v/v), hexane / EtOAc (8:2 v/v) and methanol. Other studies have used different eluents to elute cannabiniods at pH 6 by using Bond Elut Certify SPE columns. These gave acceptable recoveries from saliva, urine and plasma. For this reason, the eluents hexane / EtOAc (9:1, v/v), hexane / EtOAc (8:2, v/v) and methanol were used [164, 165].

200 µl of amfetamines working standard solution and 200 µl of cannabinoids working standard solution all at 1 µg/ml were measured into a test tube containing 30 mg of blank hair. 1 ml of 1M NaOH was added and the solution was vortex mixed. The solution was incubated at 95 °C for 10 minutes. After cooling to room temperature, 1 ml of 1 M HCl was added to neutralize and 2 ml of phosphate buffer, pH 6.0 was added. After adjusting to pH 6.0 with 1.0 M NaOH, the solution was centrifuged for 20 minutes at 2000 rpm and the supernatant transferred to a clean large vial for extraction as described in section 4.1.1.9. The acetone / chloroform step was replaced with hexane / EtOAc (9:1 v/v), hexane / EtOAc (8:2 v/v) or methanol to elute the cannabinoids. Each eluent was tested in triplicate. The solution was evaporated to dryness and derivatised as described in section 4.1.1.5.1. The deuterated working standard was added after extraction, prior to derivatisation.

4.1.1.14.1.1 Results

The recovery of Δ^9-THC did not significantly improve with the change in the eluents and also gave poor reproducibility as shown by the high % CVs. The recovery of the Δ^9-THC-COOH was approximately three times higher using eluent Hexane / EtOAc (8:2 v/v) than the original method using acetone/chloroform with good % CV reproducibility. The methanol eluent did not elute the target compounds. The recoveries of amfetamines were not affected when the cannabinoids eluent changed. The complete results are shown in Table 4.8, Table 4.9, Table 4.10, Table 4.11, Table 4.12, Table 4.13 and Table 4.14.

Table 4.8 Recovery of Δ^9-THC with different eluents at pH 6 (n=3)

Eluent	Δ^9- THC						
	Mean Peak Area Ratio		S.D		%CV		%Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Acetone / Chloroform (1:1 v/v)	0.02	0.13	0.03	0.01	14.9	4.3	12.8
Hexane / EtOAc (9:1 v/v)	0.02	0.13	0.01	0.01	35.9	4.3	13.9
Hexane / EtOAc (8:2 v/v)	0.02	0.13	0.01	0.01	28.6	4.3	14.6
Methanol	0.00	0.13	0.00	0.01	0.0	4.3	0.0

Ext: Extracted, Uext: Unextracted

Table 4.9 Recovery of Δ^9–THC-COOH with different eluents at pH 6 (n=3)

Eluent	Δ^9-THC-COOH						
	Mean Peak Area Ratio		S.D		%CV		%Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Acetone / Chloroform (1:1 v/v)	0.20	1.66	0.04	0.21	17.1	12.4	12.4
Hexane / EtOAc (9:1 v/v)	0.06	1.66	0.01	0.21	19.4	12.4	3.8
Hexane / EtOAc (8:2 v/v)	0.54	1.66	0.03	0.21	5.7	12.4	32.3
Methanol	0.00	1.66	0.00	0.21	0.0	12.4	0.0

Ext: Extracted, Uext: Unextracted

Table 4.10 Recovery of AF with different eluents at pH 6 (n=3)

Eluent	AF						
	Mean Peak Area Ratio		S.D		% CV		% Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Acetone / Chloroform (1:1 v/v)	1.97	2.56	0.23	0.29	12.9	11.3	76.9
Hexane / EtoAc (9:1 v/v))	2.09	2.56	0.25	0.29	11.9	11.3	81.5
Hexane / EtoAc (8:2 v/v)	2.01	2.56	0.11	0.29	5.6	11.3	78.5
Methanol	1.85	2.56	0.11	0.29	6.1	11.3	72.5

Ext: Extracted, Uext: Unextracted

Table 4.11 Recovery of MA with different eluents at pH 6 (n=3)

Eluent	MA						
	Mean Peak Area Ratio		S.D		% CV		% Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Acetone / Chloroform (1:1 v/v)	0.81	1.04	0.11	0.13	17.4	12.6	78.1
Hexane / EtoAc (9:1 v/v))	0.79	1.04	0.03	0.13	3.9	12.6	75.5
Hexane / EtoAc (8:2 v/v)	0.82	1.04	0.01	0.13	1.7	12.6	79.1
Methanol	0.75	1.04	0.08	0.13	10.7	12.6	71.7

Ext: Extracted, Uext: Unextracted

Table 4.12 Recovery of MDA with different eluents at pH 6 (n=3)

Eluent	MDA						
	Mean Peak Area Ratio		S.D		% CV		% Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Acetone / Chloroform (1:1 v/v)	1.03	1.35	0.12	0.11	13.0	7.9	74.6
Hexane / EtoAc (9:1 v/v))	0.97	1.35	0.05	0.11	5.3	7.9	71.7
Hexane / EtoAc (8:2 v/v)	1.04	1.35	0.02	0.11	2.0	7.9	77.0
Methanol	1.06	1.35	0.16	0.11	15.2	7.9	78.6

Ext: Extracted, Uext: Unextracted

Table 4.13 Recovery of MDMA with different eluents at pH 6 (n=3)

Eluent	MDMA						
	Mean Peak Area Ratio		S.D		% CV		% Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Acetone / Chloroform (1:1 v/v)	1.15	1.45	0.10	0.07	10.0	4.7	79.4
Hexane / EtoAc (9:1 v/v))	1.13	1.45	0.08	0.07	7.1	4.7	77.9
Hexane / EtoAc (8:2 v/v)	1.20	1.45	0.03	0.07	2.7	4.7	82.8
Methanol	1.07	1.45	0.04	0.07	4.0	4.7	74.1

Ext: Extracted, Uext: Unextracted

Table 4.14 Recovery of MDEA with different eluents at pH 6 (n=3)

Eluent	MDEA						
	Mean Peak Area Ratio		S.D		% CV		% Recovery
	Ext	Uext	Ext	Uext	Ext	Uext	
Acetone / Chloroform (1:1 v/v)	0.12	0.15	0.02	0.01	19.9	6.08	77.6
Hexane / EtoAc (9:1 v/v))	0.11	0.15	0.01	0.01	5.06	6.08	73.7
Hexane / EtoAc (8:2 v/v)	0.11	0.15	0.01	0.01	5.41	6.08	76.5
Methanol	0.11	0.15	0	0.01	4.21	6.08	72.4

Ext: Extracted, Uext: Unextracted

4.1.1.14.2 Change in Phosphate Buffer pH

A further study was carried out to investigate how the pH of the phosphate buffer affected the recovery of the cannabinoids. The pH of the phosphate buffer was changed to pH 5, 7 and 8.

200 µl of amfetamines working standard solution and 200 µl of deuterated cannabinoids working standard solution all at 1 µg/ml were measured into a test tube containing 30 mg of blank hair. 1 ml of 1M NaOH was added and the solution was vortex mixed. The solution was incubated at 95 °C for 10 minutes. After cooling to room temperature, 1 ml of 1 M HCl was added to neutralize the solution. Phosphate buffer was added at either pH 5, 6, 7 or 8. Each solution was adjusted to pH 5, 6, 7 or 8 using either 1.0 M NaOH or 1M HCl and centrifuged for 20 minutes at 2000 rpm. The supernatant was transferred to a clean large vial for extraction as described in section 4.1.1.9. The solution was evaporated to dryness and derivatised as described in section 4.1.1.5.1. Each buffer was tested in triplicate. The deuterated working standard was added after extraction, prior to derivatisation.

4.1.1.14.2.1 Results

The recoveries of the target compounds increased with increasing pH, pH 8 giving the highest recoveries. The recovery of the Δ^9-THC and Δ^9 -THC-COOH increased three and five times respectively from the original method using pH 6 and both showed satisfactory % CV reproducibility. The recoveries of amfetamines were not affected and their results demonstrated that pH 6 yielded the highest recoveries for all five amfetamines. The results are shown in Table 4.15, Table 4.16, Table 4.17, Table 4.18, Table 4.19, Table 4.20 and Table 4.21.

Table 4.15 Recovery of Δ^9-THC with varying buffer pH (n =3)

	Δ^9-THC						
	Mean Peak Area Ratio		S.D		% CV		
PH	Ext	Uext	Ext	Uext	Ext	Uext	% Recovery
5	0.08	1.5	0.02	0.11	29.7	7.1	5.3
6	0.21	1.5	0.06	0.11	27.5	7.1	13.7
7	0.14	1.5	0.05	0.11	33.7	7.1	9.0
8	0.73	1.5	0.12	0.11	16.4	7.1	49.0

Ext: Extracted, Uext: Unextracted

Table 4.16 Recovery of Δ^9 -THC-COOH with varying buffer pH (n =3)

	Δ^9 -THC-COOH						
	Mean Peak Area Ratio		S.D		% CV		
PH	Ext	Uext	Ext	Uext	Ext	Uext	% Recovery
5	0.25	2.04	0.12	0.35	48.1	17.4	12.2
6	0.26	2.04	0.05	0.35	17.2	17.4	12.9
7	0.42	2.04	0.17	0.35	40.1	17.4	20.5
8	1.26	2.04	0.03	0.35	2.6	17.4	61.8

Ext: Extracted, Uext: Unextracted

Table 4.17 Recovery of AF with varying buffer pH (n = 3)

	AF						
	Mean Peak Area Ratio		S.D		% CV		% Recovery
PH	Ext	Uext	Ext	Uext	Ext	Uext	
5	1.42	1.77	0.2	0.03	14.2	1.8	80.3
6	1.44	1.77	0.05	0.03	3.3	1.8	81.1
7	1.14	1.77	0.11	0.03	9.2	1.8	64.1
8	1.05	1.77	0.12	0.03	10.9	1.8	59.5

Ext: Extracted, Uext: Unextracted

Table 4.18 Recovery of MA with varying buffer pH (n = 3)

	MA						
	Mean Peak Area Ratio		S.D		% CV		
PH	Ext	Uext	Ext	Uext	Ext	Uext	% Recovery
5	0.55	0.66	0.10	0.03	17.5	4.8	84.4
6	0.57	0.66	0.03	0.03	5.3	4.8	87.2
7	0.48	0.66	0.15	0.03	31.3	4.8	72.7
8	0.49	0.66	0.09	0.03	17.8	4.8	75.1

Ext: Extracted, Uext: Unextracted

Table 4.19 Recovery of MDA with varying buffer pH (n = 3)

	MDA						
	Mean Peak Area Ratio		S.D		% CV		
PH	Ext	Uext	Ext	Uext	Ext	Uext	% Recovery
5	0.77	0.90	0.09	0.03	11.3	0.03	85.6
6	0.79	0.90	0.08	0.03	9.8	0.03	87.6
7	0.65	0.90	0.05	0.03	8.4	0.03	71.5
8	0.58	0.90	0.10	0.03	16.4	0.03	64.8

Ext: Extracted, Uext: Unextracted

Table 4.20 Recovery of MDMA with varying buffer pH (n = 3)

	MDMA						
	Mean Peak Area Ratio		S.D		% CV		
PH	Ext	Uext	Ext	Uext	Ext	Uext	% Recovery
5	0.89	1.14	0.13	0.05	15.0	4.2	78.0
6	0.91	1.14	0.04	0.05	3.8	4.2	80.1
7	0.80	1.14	0.06	0.05	8.1	4.2	69.8
8	0.73	1.14	0.12	0.05	15.8	4.2	64.4

Ext: Extracted, Uext: Unextracted

Table 4.21 Recovery of MDEA with varying buffer pH (n = 3)

	MDEA						
	Mean Peak Area Ratio		S.D		% CV		
PH	Ext	Uext	Ext	Uext	Ext	Uext	% Recovery
5	0.11	0.13	0.01	0.01	12.0	4.3	80.0
6	0.11	0.13	0.01	0.01	6.9	4.3	84.1
7	0.10	0.13	0.01	0.01	5.2	4.3	75.2
8	0.09	0.13	0.02	0.01	18.8	4.3	66.5

Ext: Extracted, Uext: Unextracted

4.1.1.14.3 Recovery of Cannabinoids with Phosphate Buffer pH 8 and Eluent hexane/ EtOAc (v/v)

The recovery of the cannabinoids from hair using the best eluent and best buffer pH, hexane / EtOAc (8:2 v/v) and phosphate buffer at pH 8 was investigated. The purpose of this experiment was to check if the modifications made an enhanced improvement to the method.

200 µl of amfetamines working standard solution and 200 µl of deuterated cannabinoids working standard solution all at 1 µg/ml were measured into a test tube containing 30 mg of blank hair. Alkaline pre-treatment and SPE were carried out as before with the modifications being hexane / EtOAc as eluent and pH of buffer 8. 1 ml of 1M NaOH was added and the solution was vortex mixed. The solution was incubated at 95 °C for 10 minutes. After cooling to room temperature, 1 ml of 1 M HCl was added to neutralize the solution and 2 ml of phosphate buffer, pH 8.0 was added. After adjusting with 1.0 NaOH to pH 8.0 and centrifuging for 20 minutes at 2000 rpm, the supernatant was transferred to a clean large vial for extraction as described in section 4.1.1.9. SPE was carried out as before, but hexane / EtOAc (8:2 v/v) was used instead of acetone / chloroform (1:1 v/v) as eluent. This was prepared in triplicate. The solution was evaporated to dryness and derivatised as described in section 4.1.1.5.1. The deuterated working standard was added after extraction, prior to derivatisation.

The same experiment was repeated. Standards were made up as previously described and they were measured into a test tube without the presence of hair. The purpose of this experiment was to check if there was interference from hair matrices causing the decrease in the cannabinoids recovery.

4.1.1.14.3.1 Results

The results demonstrated that a low recovery of cannabinoids still persisted in the presence of hair. The second experiment without the presence of hair showed that the cannabinoids recovery was not significantly changed. Unsatisfactory % CV reproducibility was also shown both with and without the presence of hair. The complete results are shown in Table 4.22.

Table 4.22 Recovery of cannabinoids and amfetamines with and without presence of hair (n =3)

Analyte	Mean Peak Area Ratio		S.D		% CV		% Recovery	Comment
	Ext	Unext	Ext	Unext	Ext	Unext		
Δ^9-THC	0.21	0.82	0.06	0.02	30.3	2.0	25.4	With Hair
	0.24	0.82	0.04	0.02	17.4	2.0	29.2	Without Hair
Δ^9-THC-COOH	0.05	1.65	0.02	0.06	45.8	3.5	3.2	With Hair
	0.16	1.65	0.05	0.06	34.1	3.5	9.5	Without Hair
AF	1.78	2.24	0.11	0.03	6.0	1.4	79.3	With Hair
	1.82	2.24	0.11	0.03	6.3	1.4	81.4	Without Hair
MA	0.94	1.20	0.02	0.02	2.2	1.9	78.0	With Hair
	1.01	1.20	0.08	0.02	8.2	1.9	83.9	Without Hair
MDA	1.01	1.36	0.03	0.03	2.5	2.6	74.4	With Hair
	1.06	1.36	0.04	0.03	3.4	2.6	77.9	Without Hair
MDMA	1.22	1.62	0.02	0.01	1.3	0.7	75.2	With Hair
	1.37	1.62	0.03	0.01	1.9	0.7	84.6	Without Hair
MDEA	0.95	1.20	0.04	0.03	4.4	2.1	79.8	With Hair
	1.03	1.20	0.02	0.03	2.0	2.1	85.9	Without Hair

4.1.1.14.4 Discussion and Conclusion

The original SPE method alone without the inclusion of pre-treatment methods gave acceptable recoveries for all of the compounds, AF, MA, MDA, MDMA, MDEA, Δ^9-THC and Δ^9 -THC-COOH. With alkaline pre-treatment and other pre-treatment methods the recovery of Δ^9-THC and Δ^9 -THC-COOH decreased significantly as shown by the lack of detection of lower standard concentrations (5-50 ng/30mg). The combination of alkaline pre-treatment with SPE was not a successful combination for the cannabinoids. On the other hand, the amfetamines recoveries were not affected.

Modifications to the method were made by changing the eluent and pH. The eluent change showed little improvement in recovery and unsatisfactory reproducibility for Δ^9-THC as shown by the high % CVs. The recovery of Δ^9 -THC-COOH was improved and also gave good reproducibility with Hexane / EtOAc (8:2 v/v) as the eluent compared with the original eluent, acetone/chloroform. The recoveries of Δ^9-THC and Δ^9 -THC-COOH were significantly increased with change in pH and the highest recoveries for both compounds were found at pH 8 with satisfactory reproducibility. However, the improvement was still insufficient to meet the required limits of detection.

With the best eluent and best buffer pH, hexane / EtOAc (8:2 v/v) and phosphate buffer at pH 8, the recovery of the Δ^9-THC and Δ^9 -THC-COOH were not improved. The recovery of Δ^9-THC and Δ^9 -THC-COOH with and without the presence of hair using the best eluent and best buffer pH did not show a significant change and their % CV reproducibility was also poor with and without the presence of hair. The results of this experiment demonstrated that the presence of hair did not affect the cannabinoids recovery.

The low recoveries of this method meant that it was not suitable for the detection of cannabinoids with low concentrations in hair samples, especially for the metabolite which incorporates into hair at very low concentrations. The method could possibly be improved by using a more sensitive technique such as GC-MS-NCI or GC-MS/MS [10, 134, 153, 155, 156]. Further studies are needed to investigate the influence of the combination of alkaline pre-treatment and other pre-treatment methods with SPE. Otherwise, the analysis of cannabinoids must be investigated using a separate validated method. The difficulty of Δ^9-THC and Δ^9 -THC-COOH determination in hair samples has also been reported by *Moeller et al [95]*. This method used β-glucuronidase/aryl-sulfatase followed by SPE with acetone/dichloroform (3:1) as eluent, PFPA/PFPOH as derivatising agents and analysis by GC-MS with EI detection.

4.2 Comparison of Pretreatment Methods for Amfetamine

Comparison of four pre-treatment methods, NaOH, β-Glucuronidase, HCl and MeOH was carried out for AF only. This was because there was a difficulty in obtaining hair samples that were positive for all five amfetamine compounds. An authentic case sample was used to investigate the recovery of AF by each of the methods. This sample had previously been analysed by Forensic Medicine and Science, University of Glasgow and was already known to be positive for AF.

Blank and case hair samples were decontaminated using the same procedure described in section 4.1.1.7. For standard preparation, 30 mg samples of blank hair were weighed out and spiked with 5, 10, 25, 50, 100 and 200 µl AF and 100 µl of deuterated working standard, both at 1 µg/ml to give a six point calibration curve. A blank hair sample without standard or internal standard and a blank hair sample with only 100 µl of internal standard were also prepared.

The case hair sample was cut into three segments (root - 3 cm, 3 - 6 cm and 6 - 9cm), cut into small pieces (1-2 mm) and 100 µl of AF deuterated working standard was added. Four different pre-treatment methods, described in 4.1.1.8, were used in this study. Each segment was divided into two portions and analysed by two different pre-treatment methods. It was not possible to compare all four at once because of insufficient sample. A calibration curve was also plotted for each pre-treatment method. The segment root – 3 cm was used to compare β-glucuronidase and alkaline pre-treatment. The method that gave the better recovery was then compared with methanol pre-treatment on segment 3 – 6 cm and the better of these two methods was compared with acid pre-treatment using segment 6 – 9 cm. SPE as described in section 4.1.1.9 was used to clean up the hair samples. The solution was evaporated to dryness and derivatised as described in section 4.1.1.5.1.

4.2.1 Results and Discussion

Calibration curves by each method were plotted and were shown to give good linearity and R^2 values similar to those shown previously in 4.1.1.10. The AF concentration results obtained for the case hair sample by these pre-treatment methods were calculated and they fell within a range of 0.21-0.53 ng / mg. The β-glucuronidase pre-treatment method gave a better recovery than the alkaline pre-treatment method, the methanolic pre-treatment method and the acidic pre-treatment method. The β-glucuronidase pre-treatment method was used as a reference for the other pre-treatment methods to calculate the recoveries. This method was assumed to give 100% recovery for AF and the recoveries obtained using other methods were compared to this. Alkaline pre-treatment, methanol and acid pre-treatment methods represented approximately 87%, 70% and 56%, respectively, compared with the β-glucuronidase pre-treatment method. The β-glucuronidase pre-treatment method was also found to be cleaner than either the alkaline or methanol extractions. The full scan chromatograms for blank hair samples for each pre-treatment method are shown in Figure 4.28, Figure 4.29, Figure 4.30 and Figure 4.31. The complete results for comparison are summarized in Table 4.23 and Figure 4.27.

β-Glucuronidase pre-treatment was selected as the method of choice for the extraction of AF content in hair. It was assumed that alkaline pre-treatment would give the highest recovery because this extraction completely dissolves the hair sample and all drugs are liberated from hair matrices. It has been reported that an enzyme method is suitable to extract all substances at neutral pH [166].

It has previously been reported [94] that four different pre-treatment procedures, methanol, acid (HCl 0.1 N), alkaline (NaOH 1 N) and enzymatic (β-glucuronidase/arylsulfatase) pre-treatment were compared to determine AF, MDA and MDMA using powdered positive hair samples. The clean-up method was LLE with 10 ml ethyl acetate. Derivatisation was carried out with heptafluorobutyric anhydride/ethyl acetate (2:1, v/v) and analysis by GC-MS. The results of this study demonstrated that alkaline pre-treatment gave the highest recoveries and direct methanol pre-treatment gave the lowest recoveries for all three compounds. This study concluded that the results of this comparison study are not enough to determine which extraction method is best. It was reported that factors such as the precision and practicality of each method should also be taken into account, when extraction comparison studies are carried out [70].

In another study, three pre-treatment methods were compared to extract amfetamines from hair samples. These were sodium hydroxide, methanol / HCl and methanol / trifluoroacetic acid. The highest recoveries of AF, MA, MDA, MDMA and MDEA from hair were found by acid pre-treatment with SPE extraction. In contrast, alkaline pre-treatment was found to give the best recoveries when LLE was used before SPE [92].

Acid pre-treatment and alkaline pre-treatment followed with ether extraction were compared in another study involving monkey hair. This showed that there were no significant differences in recovery between these extractions for AF and MA, but the acidic method was found to be a cleaner extract and gave lower standard deviations [97].

The results of these studies differ with the findings in this investigation which showed that the β-glucuronidase method gave the highest recovery of AF. Although, alkaline pre-treatment has the ability to liberate the drugs from the hair sample it requires extensive clean up procedures to eliminate hair matrix substances which can cause interference with the analysis. This has been highlighted in the previously mentioned article where both LLE and SPE were used following alkaline pre-treatment [92]. Obtaining good recoveries with alkaline pre-treatment is dependent on having good purification of the hair digest to eliminate more interfering substances. It would appear that the combination of a pre-

treatment step and a particular clean-up method may significantly affect the recovery. For instance, alkaline pre-treatment with LLE may produce the highest recovery compared with other pre-treatment methods with the same LLE. However, another type of pre-treatment method may be better with SPE than the alkaline pre-treatment with the same SPE method [94].

Other factors that need to be considered when choosing a method are the cost and time of sample preparation. Extensive clean-up procedures may significantly increase both of these factors. It was reported that there are important considerations which should be kept in mind when selecting the pre-treatment procedures such as type of analyte and its stability, distribution ratio between analyte and its metabolites and finally, subsequent analysis (qualitative or quantitative) [167].

Table 4.23 The concentration of AF measured in different segments of the authentic hair sample

Methods	Concentration ng/mg	Segment	Percentage
Alkaline	0.46	Root - 3 cm	87%
Enzymatic	0.53	Root - 3 cm	100%
Methanol	0.21	3 - 6 cm	70%
Enzymatic	0.3	3 - 6 cm	100%
Acid	0.26	6 -9 cm	56%
Enzymatic	0.46	6 -9 cm	100%

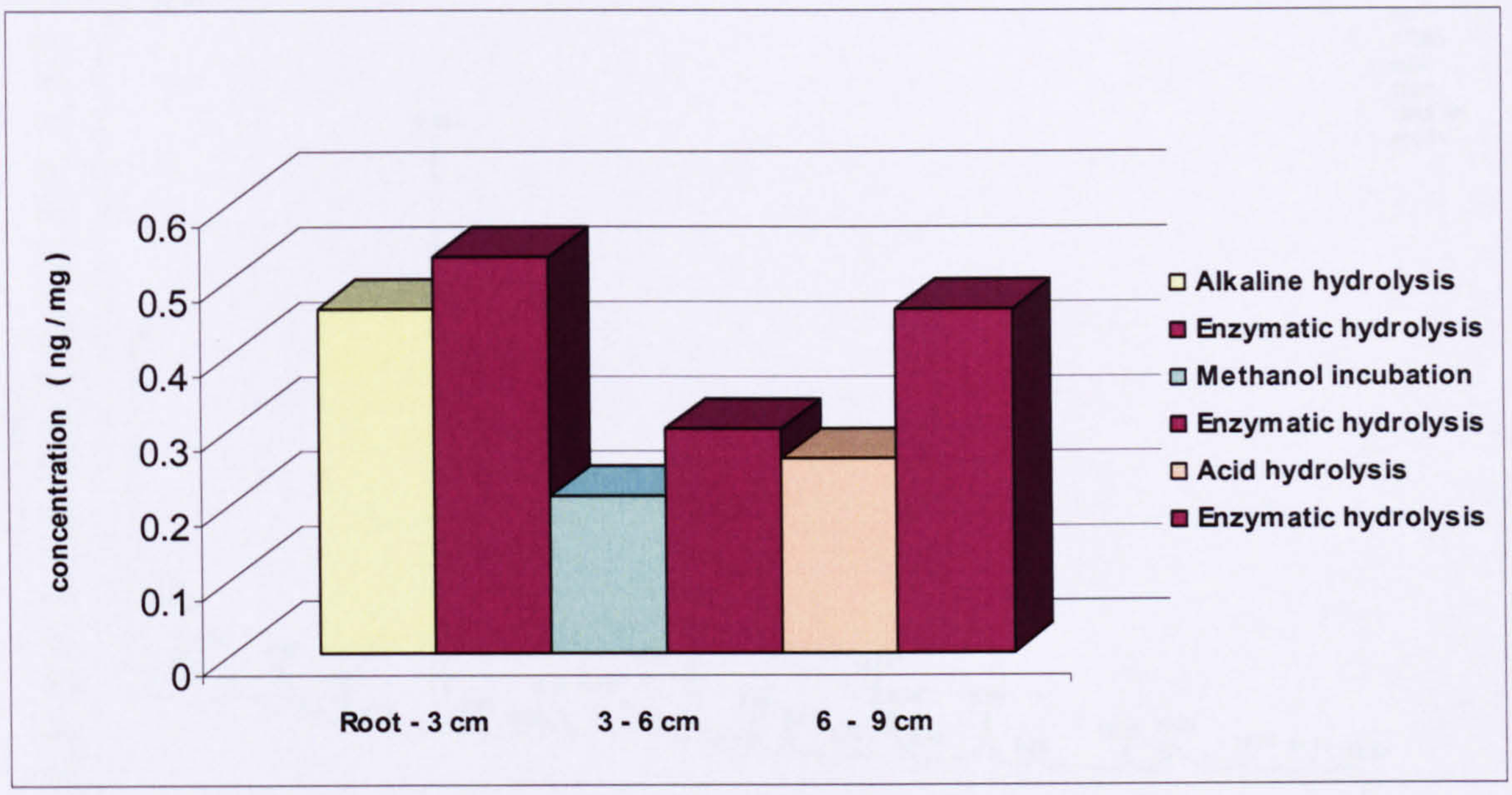


Figure 4.27 The concentration of AF in different segments of the authentic hair sample

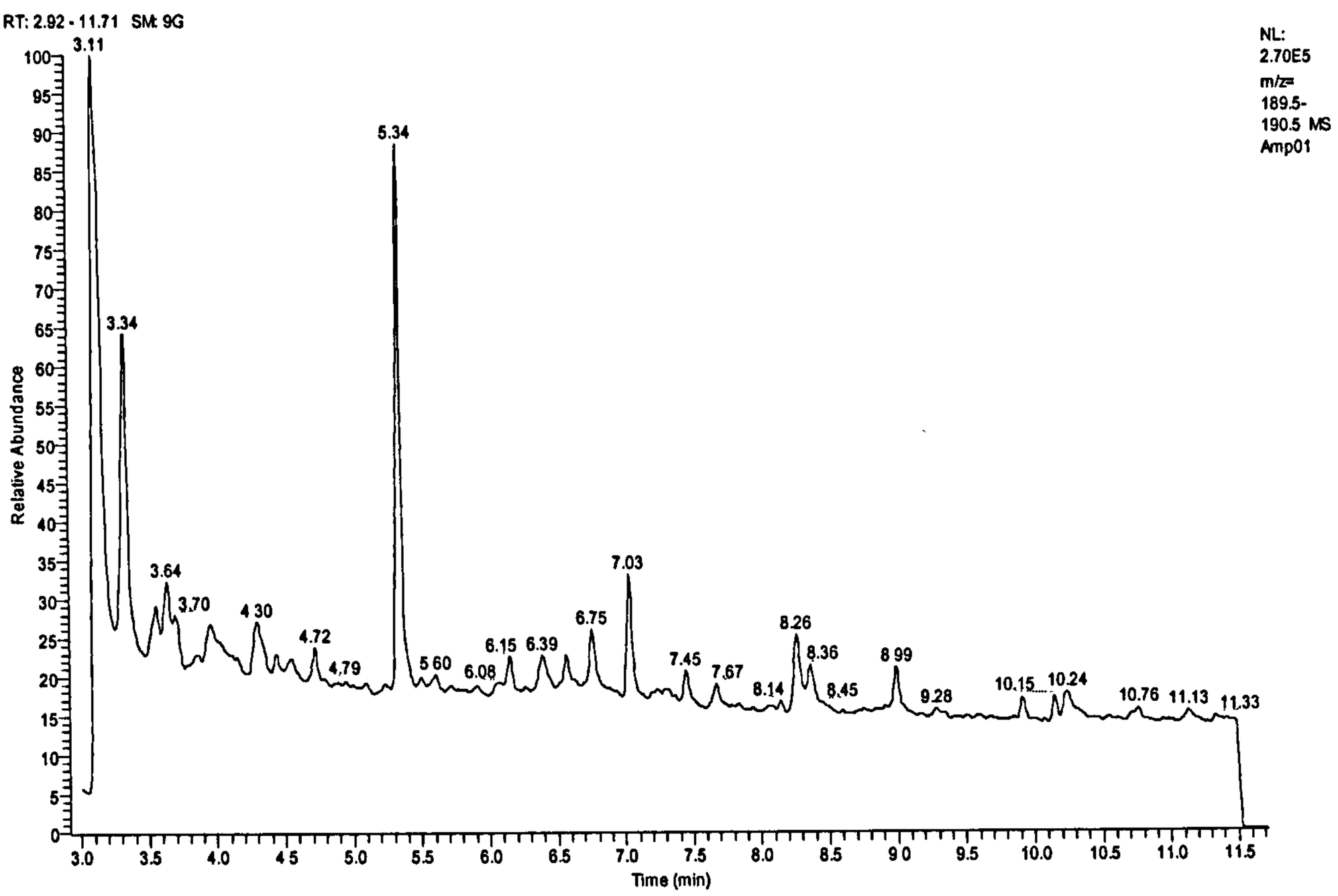


Figure 4.28 Full Scan Chromatogram of blank hair sample after enzymatic pre-treatment

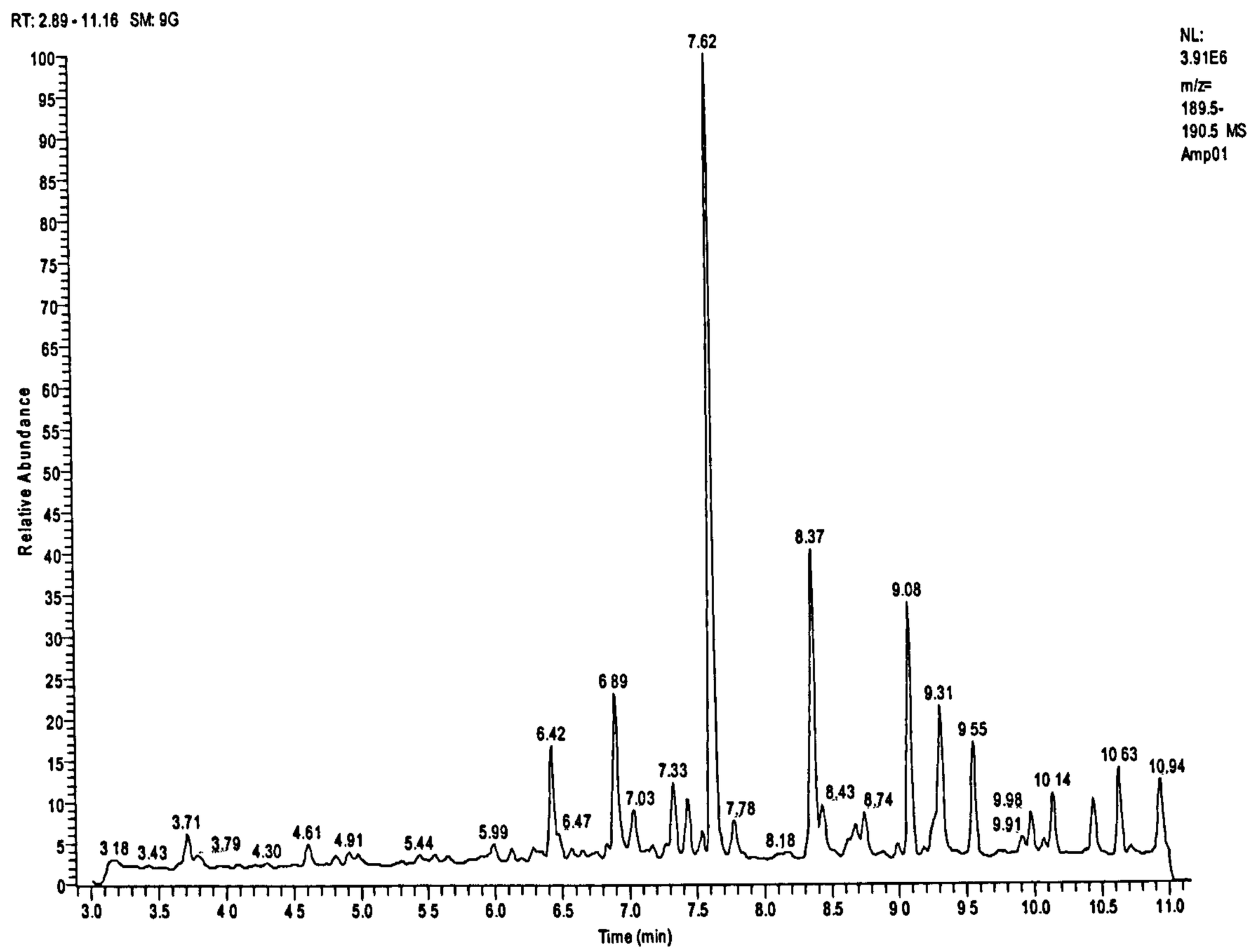


Figure 4.29 Full Scan Chromatogram of blank hair sample after acid pre-treatment

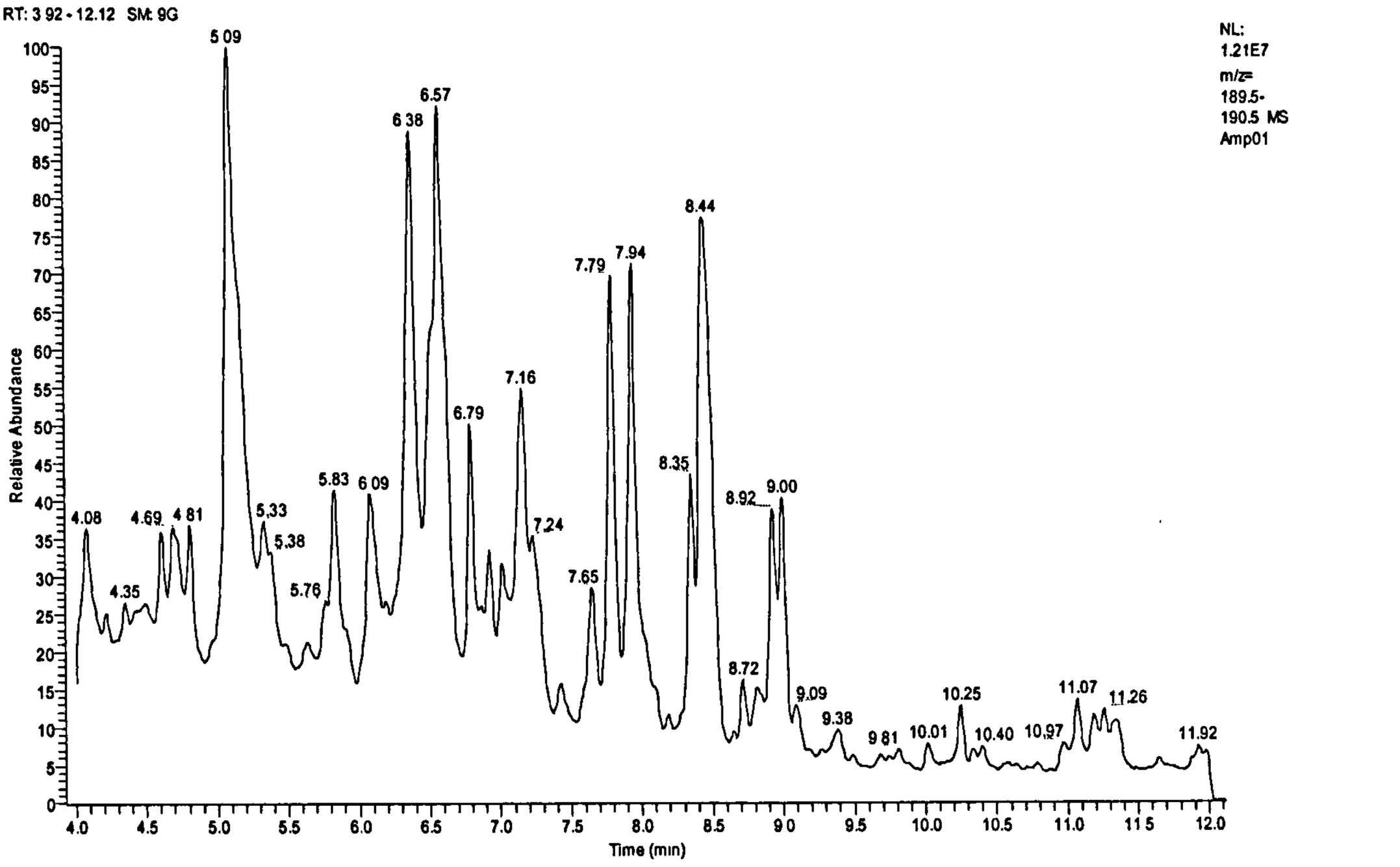


Figure 4.30 Full Scan Chromatogram of hair blank sample after alkaline pre-treatment

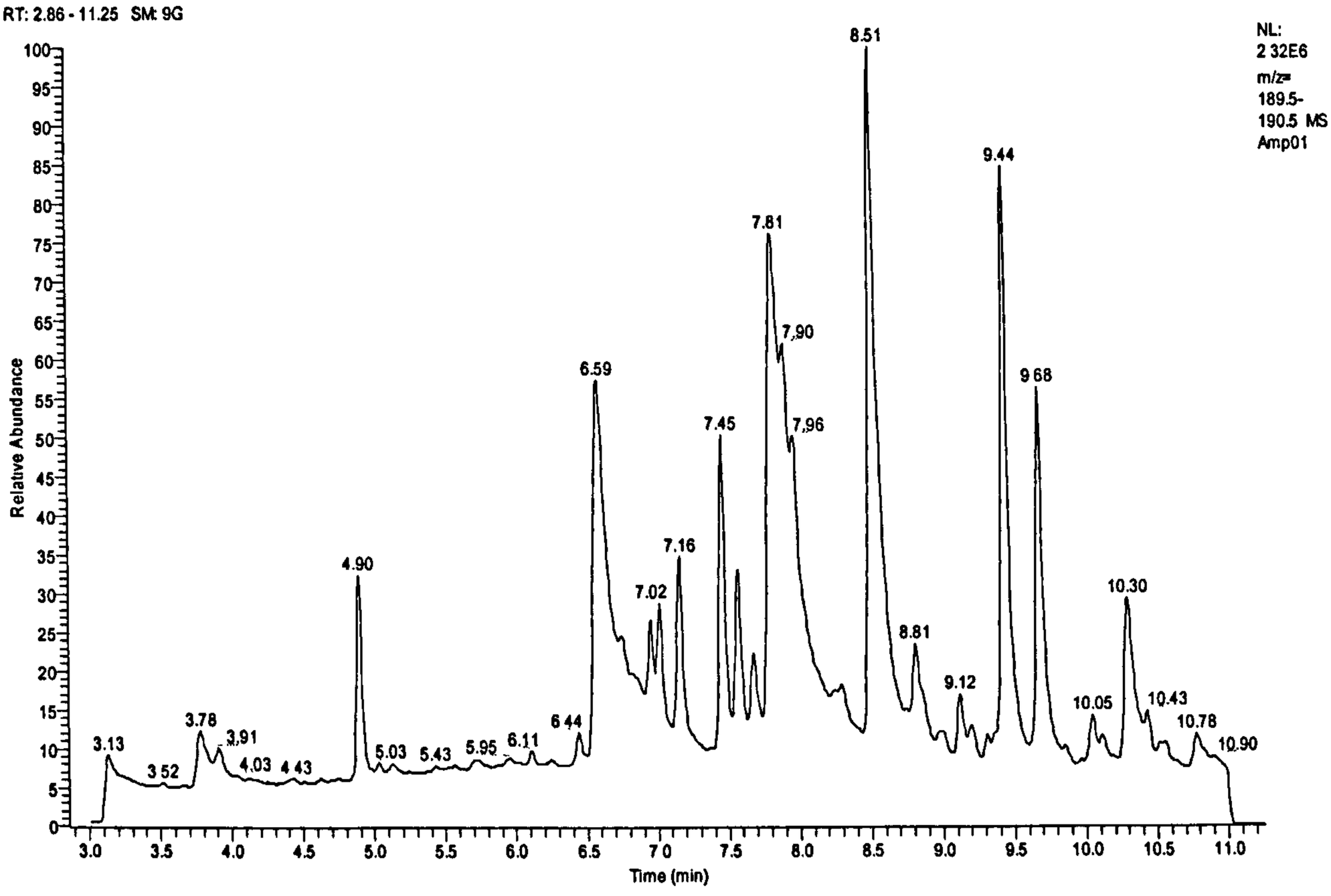


Figure 4.31 Full Scan Chromatogram of blank hair sample after methanolic pre-tretment

4.3 Method validation

In accordance with the results of the comparative study in section 4.2, the best recovery for AF was obtained by the β-glucuronidase pre-treatment method. This method as described in 4.1.1.8 was used and validated for amfetamines in hair.

Hair samples were decontaminated using the same procedure described in section 4.1.1.7, the solutions were extracted using the SPE method as described in section 4.1.1.9 and the solution was evaporated to dryness and derivatised as described in section 4.1.1.5.1.

4.3.1 Linearity

Linearity was demonstrated by plotting the calibration curves of five amfetamines. Standards were prepared by weighing 30 mg of blank hair into a test tube and spiking with 5, 10, 25, 50, 100 and 200 µl of a 1µg/ml working standard solution containing AF, MA, MDA, MDMA and MDEA. 100 µl of deuterated internal standard at 1µg/ml was added to every standard to give a six point calibration curve. A blank hair sample without standard or internal standard and a blank hair sample with only 100 µl of internal standard were also prepared. Calibration curves were plotted using concentration against PAR (drug peak area / internal standard peak area) of each amfetamine. The results are presented in Table 4.24, Table 4.25 and Figure 4.32.

4.3.2 Recovery

The recoveries were determined and calculated at low, medium and high concentrations for each of the five amfetamines, by taking the mean PAR of extracted samples divided by the mean PAR of the unextracted standards at the equivalent concentration and multiplying by 100. Spiked hair samples (30 mg) were prepared in five replicates at three different concentrations (10, 50 and 200 µl of a 1µg/ml standard). Unextracted standards were prepared at the same time as spiked hair samples and kept in the fridge while the extraction was carried out. Deuterated internal standards (100 µl of a 1µg/ml standard) were added to extracts and unextracted standards after the extraction. The results are presented in Table 4.26.

4.3.3 Intra-day and Inter-day Precision

4.3.3.1 Intra-day precision between extracts

The intra-day precision between extracts was carried out in five replicates. Spiked hair samples (30 mg) were prepared on the same day at three different concentrations (10, 50 and 200 µl of a 1µg/ml standard) in the presence of deuterated internal standard (100 µl of a 1µg/ml standard). The results are presented in Table 4.27.

4.3.3.2 Intra-day precision between injections

The intra-day precision between injections was carried out by injecting the same standard five times. Spiked hair samples (30 mg) were at three different concentrations (10, 50 and 200 µl of a 1µg/ml standard) in the presence of deuterated internal standard (100 µl of a 1µg/ml standard. The results are presented in Table 4.28.

4.3.4 Inter-day Precision

Inter-day precision was determined by using spiked hair samples (30 mg) at three different concentrations (10, 50 and 200 µl of a 1µg/ml standard) in the presence of deuterated internal standard (100 µl of a 1µg/ml standard) over a period of five days. The results are presented in Table 4.29.

4.3.5 Limits of Quantitation and Detection

The limits of quantitation (LOQ) and detection (LOD) were calculated from the intercept and the standard error of the regression line. The equations used for calculation of LOQ and LOD are shown in the equations 1 and 2 for LOQ and the equations 3 and 4 for LOD, where y_B is intercept, s_B is the standard error of the regression line and m is the gradient. The calibration curves of all five amfetamines were plotted, by spiking blank hair samples (30 mg) with amfetamine standards to obtain concentrations of 0.5, 0.75, 2, 5, 10 and 25 ng/30 mg of hair and adding 100 µl of a 1µg/ml deuterated internal standard. A blank hair sample without standard or internal standard and a blank hair sample with only 100 µl of internal standard were also prepared and extracted. The limit of detection is defined as the intercept plus three standard errors of the regression line and the limit of quantitation is

defined as intercept plus five standard errors of the the regression line [168]. The limits of detection and quantitation for all amfetamines are presented in Table 4.30.

$$y_{LOQ} = y_B + 5S_B \qquad \text{Equation 1}$$

$$L.O.Q = \frac{(y_{LOQ} - y_B)}{m} \qquad \text{Equation 2}$$

$$y_{LOD} = y_B + 3S_B \qquad \text{Equation 3}$$

$$L.O.D = \frac{(y_{LOD} - y_B)}{m} \qquad \text{Equation 4}$$

Table 4.24 Calibration concentration and peak area ratio for extracted amfetamines

Concentration (ng/30mg)	AF/AF-d_5 190/194	MA/MA-d_5 204/208	MDA/MDA-d_5 325/330	MDMA/MDMA-d_5 339/344	MDEA/MDEA-d_5 218/223
5	0.07	0.04	0.09	0.07	0.01
10	0.15	0.08	0.16	0.15	0.02
25	0.34	0.19	0.34	0.29	0.05
50	0.65	0.4	0.67	0.68	0.09
100	1.22	0.75	1.21	1.25	0.18
200	2.45	1.48	2.47	2.49	0.35

Table 4.25 Linear regression values

Analyte	R^2	Intercept	Slope
AF	1.000	0.0267	0.0121
MA	0.996	0.0109	0.0074
MDA	0.999	0.0360	0.0121
MDMA	0.999	0.0164	0.0124
MDEA	1.000	0.0037	0.0017

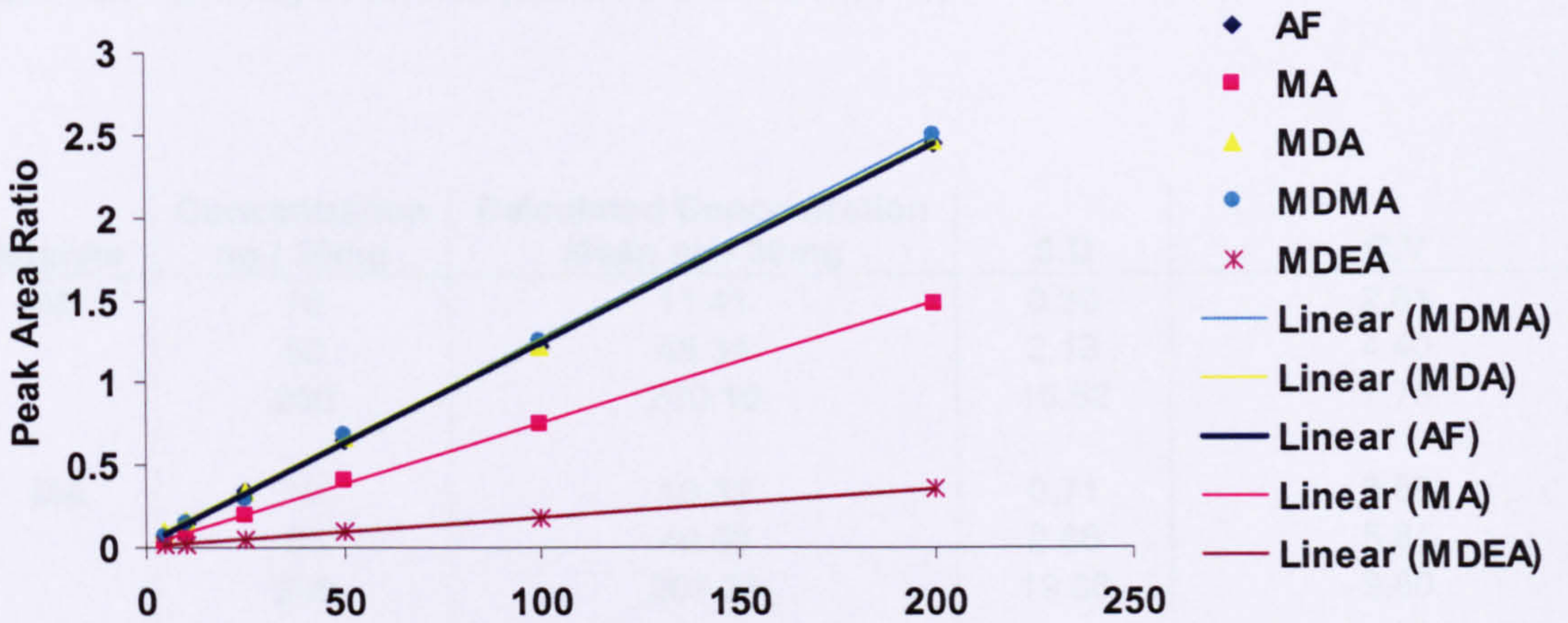


Figure 4.32 Amfetamines Calibration curves (AF, MA, MDA, MDMA and MDEA, 0 to 200 ng)

Table 4.26 Recovery results for the extracted Amfetamines from hair (n=5)

Analyte	Concentration (ng)	Mean Peak Area Ratio		S.D		% CV		% Recovery
		Ext	Unext	Ext	Unext	Ext	Unext	
AF	10	0.09	0.11	0.00	0.01	4.82	7.07	84.2
	50	0.43	0.57	0.02	0.02	4.65	3.08	76.0
	200	1.65	2.35	0.01	0.08	0.86	3.56	71.4
MA	10	0.08	0.11	0.00	0.00	4.36	4.31	77.7
	50	0.39	0.53	0.02	0.02	5.6	3.55	74.2
	200	1.58	1.80	0.11	0.10	7.27	5.55	87.7
MDA	10	0.11	0.13	0.00	0.01	4.23	4.69	79.6
	50	0.49	0.65	0.01	0.02	2.42	3.12	75.7
	200	2.12	2.74	0.10	0.09	4.82	3.44	77.3
MDMA	10	0.01	0.18	0.01	0.01	5.75	5.77	76.0
	50	0.66	0.87	0.01	0.03	2.01	3.2	76.2
	200	2.89	3.72	0.14	0.15	4.83	3.94	77.6
MDEA	10	0.01	0.02	0.00	0.00	1.33	6.77	82.6
	50	0.07	0.08	0.00	0.00	5.38	3.1	85.2
	200	0.28	0.31	0.02	0.01	6.30	3.68	88.4

Table 4.27 Intra-day Precision (between extraction, n=5)

Analyte	Concentration ng / 30mg	Calculated Concentration Mean ng / 30mg	S.D	C.V
AF	10	11.41	0.30	2.61
	50	48.34	2.13	4.40
	200	200.10	15.52	7.76
MA	10	10.32	0.71	6.86
	50	49.53	2.88	5.81
	200	207.28	19.88	9.60
MDA	10	10.53	0.50	4.74
	50	48.53	2.53	5.34
	200	199.97	11.66	5.83
MDMA	10	10.93	1.04	9.52
	50	46.74	3.87	8.29
	200	200.00	9.04	4.52
MDEA	10	13.28	0.53	3.97
	50	49.00	2.08	4.24
	200	196.62	10.91	5.55

Table 4.28 Intra-day Precision (between injection, n=5)

Analyte	Concentration ng/ 30mg	Calculated Concentration Mean ng / 30mg	S.D	C.V
AF	10	10.75	0.75	7.01
	50	49.12	0.40	0.81
	200	200.88	2.37	1.18
MA	10	9.04	0.34	3.75
	50	50.77	0.46	0.91
	200	199.52	2.32	1.16
MDA	10	12.46	0.57	4.57
	50	46.74	2.61	5.58
	200	201.55	14.88	7.38
MDMA	10	10.07	0.58	5.78
	50	49.92	3.92	7.85
	200	199.74	2.71	1.36
MDEA	10	14.34	0.37	2.56
	50	49.14	0.41	0.84
	200	203.14	1.57	0.77

Table 4.29 Inter-day Precision (n=5)

Analyte	Concentration ng / 30mg	Calculated Concentration Mean ng / 30mg	S.D	C.V
AF	10	11.27	1.76	15.6
	50	47.36	4.29	9.0
	200	201.95	2.01	1.0
MA	10	11.09	2.06	18.5
	50	47.80	5.22	10.9
	200	203.47	5.24	2.5
MDA	10	11.07	2.13	19.2
	50	49.48	4.43	8.9
	200	202.45	3.39	1.6
MDMA	10	11.34	2.09	18.4
	50	48.73	3.31	6.8
	200	202.12	2.34	1.1
MDEA	10	10.90	2.02	18.5
	50	46.28	3.32	7.1
	200	202.31	1.97	0.9

Ext: Extracted, Uext: Unextracted

Table 4.30 Limit of Quantitation and Detection

	AF (ng/30mg)	MA (ng/30mg)	MDA (ng/30mg)	MDMA (ng/30mg)	MDEA (ng/30mg)
LOD	2.58	0.70	2.19	2.33	1.36
LOQ	4.30	1.17	3.89	3.65	2.25

4.3.6 Results and Conclusion

The method validation was carried out for linearity, recoveries, intra and inter-day precision, limit of quantitation (LOQ) and limit of detection (LOD) for all amfetamines compounds. Linearity was obtained from 5 to 200 ng/30mg of hair with correlation coefficients (R^2) ranging from 0.999-1.000 for all amfetamines. The recovery for all five amfetamines was higher than 71%. Coefficients of variation for the intra-day precision between extracts and between injections were all less than 10 % at each concentration for all five amfetamine compounds. Coefficients of variation for the inter-day precision were

all less than 15 % at each concentration for all five amfetamine compounds. The limits of detection and quantitation for all five amfetamines were between 0.7 to 2.58 ng/30mg and 1.17 to 4.3 ng/30mg, respectively. The method validation results showed good validation data for all five amfetamines and indicated that this method is adequate for the purpose of the amfetamines analysis in hair with satisfactory sensitivity. Consequently, this method was applied to 22 case hair samples to measure the concentration of amfetamines (Chapter 6).

5 Stability of Amfetamine in Hair Samples

5.1 Introduction

The stability of drugs in biological fluids may be affected by different factors such as temperature, storage time, drug concentration, sample, pH, the type of biological fluid, the use of preservatives and the method of storage. All of these can lead to a significant change in the analyte concentration within the specimen and can lead to misinterpretation of quantitative results [169]. Consequently, the forensic toxicologist must consider the limited stability of specimens to give the proper interpretation of analytical results. For instance chemical degradation, ongoing enzymatic metabolism and redistribution happens in postmortem specimens between the death and the sampling of matrices [80]. It has been reported that all drug concentrations can change in postmortem specimens and that the change varies between drugs [125].

The stability of MDA, MDMA and MDEA in pooled serum, whole blood, water and urine samples whilst stored at different temperatures (-20, 4 or 20 °C) in the dark over a period of 21 weeks has been investigated. It was reported that there was no degradation. Therefore, MDA, MDMA or MDEA samples stored for several weeks (13 weeks for whole blood and ≥21 weeks for the other matrices, water, serum and urine) are appropriate to analyse. Severe matrix degradation was found for whole blood and serum stored at room temperature which could lead to undetectable concentrations of the drugs, particularly where the initial concentrations are low [80].

The stability of drugs (methoxyphenamine, as model compound for methamfetamine) in hair samples was described. Drug levels in hair were measured in a living subject over a period of 6 months. The results obtained from this study revealed that the drug decreased about 50% after 5-6 months. This decrease is likely to be a result of drug being removed from hair through normal washing and possibly the decomposition of some of the drug with time [170].

The stability of opiates in hair was investigated after cosmetic treatment (bleaching and permanent waving) of hair spiked with opiates and authentic hair samples known to be positive for opiates. The results showed a decrease in the concentration of spiked hair samples that ranged from 82 % to 98 % after bleaching and ranged from 70 % to 80 % after permanent waving. The drug concentration also decreased for authentic hair samples,

but was not as significant. Exposure of human hair scalp to sunlight and cosmetic treatment was also shown to cause morphological alterations in the hair fibres affecting the drug concentrations [171].

It has been reported that the opiates were significantly decreased or even lost when hair samples were exposed to unfavourable environmental conditions such as water and soil for various time intervals [172].

Although a number of analytical methods have been reported for the analysis of AF in hair samples as mentioned previously in section 3.1.6, none of these methods have reported on the stability of AF in hair in cases of drowning through immersion in either sea or fresh water. For this study, the effect of sea water and fresh water on the concentration of AF in hair specimens was investigated.

5.2 Aim

The aim of this study was to assess the validity of analysing hair samples for the presence of AF in victims of drowning.

5.3 Experimental

5.3.1 Hair and Water Collection

AF positive hair samples were collected from volunteers, with known AF drug abuse history. This was assessed using medical files. In many of these cases urine samples had been previously analysed by the laboratory at the Alamal Medical Complex in Riyadh prior to subjects entering rehabilitation programs. The majority of these samples were found to be positive for AF. Hair was obtained by cutting from the area at the back of the head (posterior vertex) and as close to the scalp as possible. Once collected, a hair sample was placed in aluminium foil, with the scalp end clearly identified, sealed in a paper envelope and stored at room temperature until use.

The fresh and sea water were obtained from Loch Lomond and from the River Clyde Estuary, Gourock near Glasgow in Scotland, respectively.

5.3.2 Stability of Amfetamine in Hair Samples Submerged in Fresh Water and Sea Water

5.3.2.1 Initial study for Amfetamine Stability

A preliminary experiment was carried out to assess the stability of AF in hair samples which had been submerged in fresh and sea water for different periods of time.

In order to prepare hair standards, 30 mg of blank hair samples were weighed out and spiked with 5, 10, 25, 50, 100 and 200 µl of AF and 100 µl of deuterated working standard, both at 1 µg/ml to give a six point calibration curve. A blank hair sample without standard or internal standard and a blank hair sample with only 100 µl of internal standard at 1 µg/ml were also prepared and extracted.

A hair positive specimen was cut to give 0 – 3 cm length and this was separated into 7 portions. One of these portions was stored as an original specimen and 3 portions were immersed in 5 ml of sea water and the other 3 portions were immersed in 5 ml of fresh water for 1 day, 1 week or 2 weeks at 5 °C. Hair samples were analysed using the validated method described in section 4.3.

5.3.2.1.1 Initial Study Results

All wash fraction results were negative for AF. A calibration curve was plotted for AF and was shown to give good linearity and R^2 values similar to that shown previously in section 4.1.1.10.

The preliminary results obtained in this study showed that the AF hair concentration significantly decreased with the length of time in sea water, while the AF hair concentrations showed no obvious change in fresh water. The decrease of hair AF concentration in sea water was approximately 40% after one day, 69% after one week and 84% after two weeks. The complete results for the AF concentrations found in the hair samples are summarised in Figure 5.1 and Table 5.1.

The preliminary data from this study showed that there was an effect on the concentration of AF in hair when submerged in different types of water (fresh and sea). These results led to a further investigation to assess the stability of AF in hair samples submerged in these types of water.

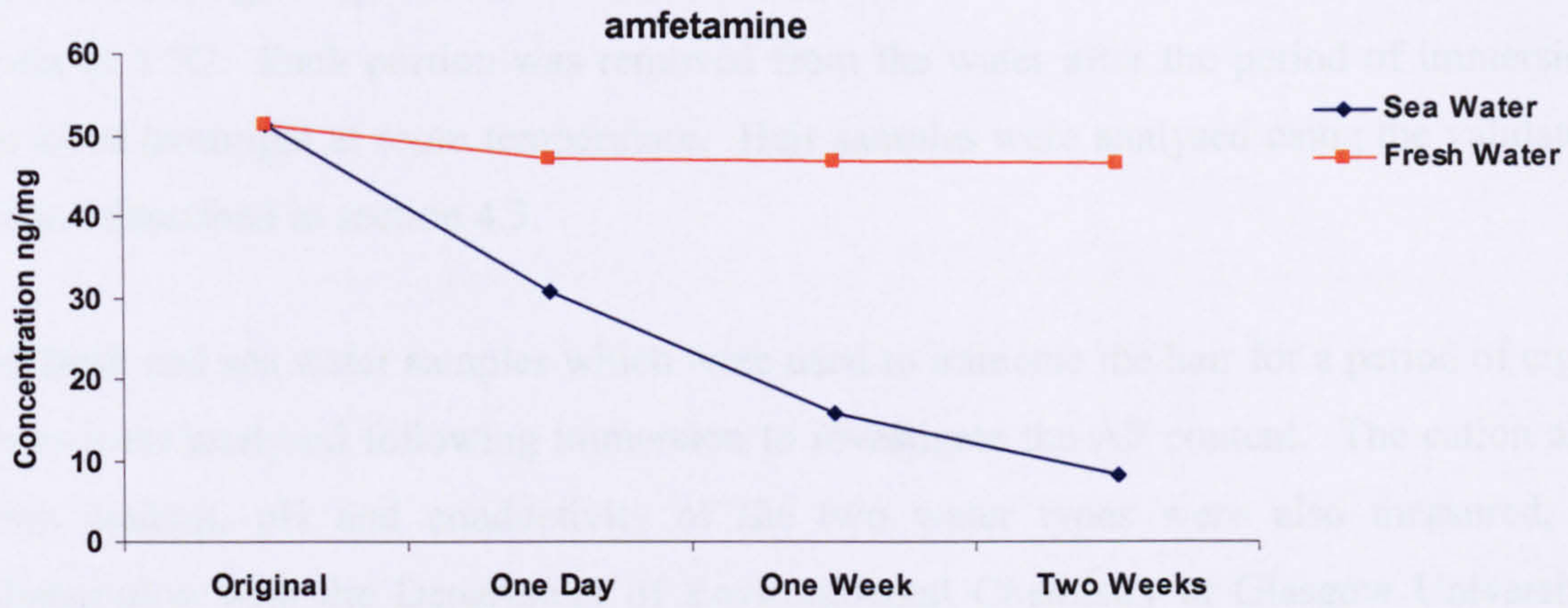


Figure 5.1 Effect of sea and fresh water on AF concentration

Table 5.1 Concentration of AF in hair after immersion in sea and fresh water (ng/mg)

Incubation Time	Sea Water (ng/mg)	Fresh Water (ng/mg)
Original	51.3	51.3
One Day	30.8	46.9
One Week	15.8	46.7
Two Weeks	8.1	46.6

5.3.2.2 Further Investigation into the Stability of Amfetamine in Hair Samples

Hair standards were prepared as in section 5.3.2.1. Ten hair samples from known AF users were collected and used to assess the stability of AF in hair samples which had been submerged in fresh or sea water for a period time. Fresh water and sea water were analysed for AF using the β-glucuronidase validated method described in section 4.3. This was to ensure that the water samples were negative and that any possibility of contamination from the water could be excluded.

Hair positive specimens were cut to give 0–3 cm length. Six of these specimens were separated into 8 portions each. One of these portions was stored as an original specimen and 7 portions were immersed in 5 ml of sea water for 1, 2 days, 1, 2, 3, 4 or 8 weeks at 5 °C. The other four specimens were separated into 5 portions, one of these portions was

stored as an original specimen and 4 were immersed in 5 ml of fresh water for 1, 2, 4 or 8 weeks at 5 °C. Each portion was removed from the water after the period of immersion and dried overnight at room temperature. Hair samples were analysed using the validated method described in section 4.3.

The fresh and sea water samples which were used to immerse the hair for a period of eight weeks were analysed following immersion to investigate the AF content. The cation and anion content, pH and conductivity of the two water types were also measured, in collaboration with the Department of Environmental Chemistry at Glasgow University. Calcium (Ca^{2+}), Magnesium (Mg^{2+}), Sodium (Na^{+}), Potassium (K^{+}), Chloride(Cl^{-}) and Sulphate (SO_4^{2-}), conductivity, pH were measured using an Atomic Absorption spectrophotometer, Flame Photometer, Ion Chromatography, Conductivity Meter and pH Meter, respectively.

5.3.2.2.1 Result and Discussion

A calibration curve was plotted for AF and was shown to give good linearity and R^2 values similar to that shown previously in section 4.1.1.10. For hair samples incubated in salt water, DCM wash fraction 3 was found to be positive in 24 out of 48 segments. DCM wash fraction 4 was found to be negative for all segments. For hair samples incubated in fresh water, only 2 out of 21 segments were found to be positive. These were very low positives, so these segments did not require further washing. Washing the hair in salt water would appear to remove some of the drug incorporated through use. It is possible that soaking in salt water may increase the porosity of the cuticle compared with soaking in fresh water. The wash fraction results are listed in Table 5.2 and Table 5.3

Fresh water and sea water samples analysed prior to the immersion of hair were determined to be negative for AF.

The initial concentrations of the hair samples investigated ranged from 0.17 to 57.69 ng/mg of hair. Hair samples that were submerged in sea water showed a significant decrease of AF concentration with time compared with hair samples that were submerged in fresh water. The decrease in hair AF concentration after one day in sea water ranged from 0.1-85% and after eight weeks ranged from 88-97%. In fresh water, after one week the concentration was found to decrease by 9-17% and after eight weeks by 16-60%. The complete results for the AF concentrations found in the hair samples are given in Table 5.4 and Table 5.5.

Figure 5.2 and Figure 5.3 show the general decrease in hair AF concentration with time for immersion in sea and fresh water respectively. In Figure 5.2 the log concentration was plotted against time.

The sea and fresh water samples which were used to immerse the hair for a period of eight weeks were found to be positive for the unchanged drug. The original total amount of drug in the hair sample (ng) was calculated by multiplying the original concentration in hair (ng/mg) by the weight of the hair used. The complete results are given in Table 5.6 and Table 5.7. These Tables show the amount of drug washed out as a percentage of the amount of drug found in the water sample compared to the amount extracted from hair. In all six sea water cases and in two out of four fresh water cases this % was greater than 100 %. This would tend to show that the true recovery of AF from hair is much lower than the recovery of AF from spiked hair samples.

The pH measurements of sea and fresh water were 8.1 and 7.0 respectively. The conductivity of sea and fresh water samples were 615 and 0.94 mS respectively. The cation and anion content of the two water types are given in Table 5.8.

The results demonstrated a faster decrease of AF concentration in sea water than in fresh water. A possible explanation could be the displacement effect of metal ions in the water (especially cations) with AF. For example, keratin and melanin present in hair have potential binding sites for binding drugs and metals [173], such as, inorganic metal cations, neutral organic compounds, and organic cations [37]. Binding with these substances can be selective [35] and the affinity of the interaction can be varied depending on the substance [38]. The exact interaction of melanin with these substances is still unknown [174].

The presence of cystine in human hair, which represents approximately 14% of the structure is largely responsible for the high affinity of hair for metals. The metals found in hair are bound either to sulphur atoms in cystine or to sulphydryl groups present in other amino acids.

Melanins are polyanionic polymers containing negatively charged carboxyl groups and semiquinones at physiological pH. Therefore cations can bind with melanin by ionic interaction. Moreover, organic amines and metal ions are positively charged at physiological pH and bind with melanin by electrostatic forces between their cationic groups and the negative charges in the melanin polymer. Van der waal`s attractions also

enhance the ionic binding. Also, uncharged metals such as mercury, may also bind to the hydrophobic core of the melanin in the hair [175]

The competition between drugs to melanin binding sites has been reported. The binding of desipramine to melanin was reduced in the presence of haloperidol. This study showed that these drugs may use similar binding sites in melanin and are competitive for these. [176]

The binding of Mg(II), Ca(II), Zn(II), Cu(II) and Fe(III) to Ethylenediaminetetraacetic acid (EDTA)-washed melanin, obtained from cuttlefish, was examined by enriching melanin with these metal ion solutions at room temperature overnight. It was reported that eumelanin possesses three types of functional groups which can act as binding sites to metal cations, carboxylic acid, hydroxyl and amine groups. It was concluded that Mg(II), Ca(II), Zn(II) bind to carboxylic acid groups, Cu(II) binds to hydroxyl(OH) groups and Fe(III) binds to hydroxyl or amine groups. Binding of metal cations with carboxylic acid will replace Na+ which is the bound metal cation in (EDTA)-washed melanin, while binding to hydroxyl and amine groups replaces the proton (H^+), consequently, the pH will changed by releasing protons in solutions [177]

The determination of the relative melanin-affinity of metal ions was assessed by the ability of the metal ions to compete with paraquat for binding to melanin. It was reported that the affinity of the alkali metals and alkaline earth metals was $Cs^+>Rb^+>K^+>Na^+>Li^+$ and $Ba^{2+}>Sr^{2+}>Ca^{2+}>Mg^{2+}$ respectively. The presence of the metal ions will decrease and inhibit of the parquet binding to melanin [178].

Melanin therefore may behave as a weak cationic exchange polymer and may bind with cationic drugs such as AF both ionically and covalently [179, 180]. Amfetamine is a polar drug and binds with polar functional groups such as hydroxyl, carboxylic acid, and amino groups in melanin [181].

It has been reported that while metal ions such as Na^+, Ca^{2+}, K^+ may compete for binding sites in melanin and inhibit cationic drugs from binding with melanin, especially when competing for the same binding sites, anions did not effect this binding [182]. Consequently, the high concentration of cations in the sea water may explain the increased rate of removal of AF from hair when compared to fresh water.

The sea and fresh water samples used to incubate the hair were extracted by SPE and analyzed after eight weeks of incubation. The analyses showed the presence of AF in all samples at reducing concentrations with time due to the removal of the AF from hair to water. The unchanged drug was found in the water samples. Interestingly, more drug was recovered in the water than expected indicating that the analytical method used to extract AF from the real hair sample has a much lower recovery than indicated by the use of spiked standards.

It has been shown that the interpretation of drugs found in hair samples obtained from victims of drowning require careful interpretation. Sea water has the potential to cause the complete loss of drug from hair with increased time, while, fresh water may cause partial or possible complete loss with increased time.

Table 5.2 Concentration of AF in wash fractions for the samples submerged in sea water

Sample	Incubation time	AF Concentration in Wash Fractions (ng/mg)	
		DCM Wash Fraction	DCM Wash Fraction 4
Sea Water, Hair 05	Original	14.61	0.00
	One day	1.80	0.00
	Two day	0.57	0.00
	One week	0.34	0.00
	Two week	0.07	0.00
	Three week	0.43	0.00
	Four week	0.25	0.00
	Eight week	0.00	0.00
Sea Water, Hair 08	Original	0.43	0.00
	One day	0.03	0.00
	Two day	0.00	0.00
	One week	0.00	0.00
	Two week	0.00	0.00
	Three week	0.00	0.00
	Four week	0.00	0.00
	Eight week	0.00	0.00
Sea Water, Hair 10	Original	0.53	0.00
	One day	0.96	0.00
	Two day	0.80	0.00
	One week	0.00	0.00
	Two week	0.00	0.00
	Three week	0.00	0.00
	Four week	0.00	0.00
	Eight week	0.00	0.00
Sea Water, Hair 12	Original	0.00	0.00
	One day	0.40	0.00
	Two day	0.00	0.00
	One week	0.00	0.00
	Two week	0.00	0.00
	Three week	0.00	0.00
	Four week	0.00	0.00
	Eight week	0.00	0.00
Sea Water, Hair 16	Original	6.50	0.00
	One day	0.00	0.00
	Two day	1.12	0.00
	One week	0.00	0.00
	Two week	0.15	0.00
	Three week	0.26	0.00
	Four week	0.11	0.00
	Eight week	0.00	0.00
Sea Water, Hair 17	Original	31.99	0.00
	One day	19.35	0.00
	Two day	0.22	0.00
	One week	0.00	0.00
	Two week	7.09	0.00
	Three week	0.47	0.00
	Four week	1.26	0.00
	Eight week	0.00	0.00

Table 5.3 Concentration of AF in wash fraction for the samples submerged in fresh water

Sample No.	Incubation time	AF Concentration in Wash Fractions (ng/mg)
		DCM Wash Fraction 3
Fresh Water, Hair 04	Original	0.00
	One week	0.00
	Two week	0.00
	Four week	0.00
	Eight week	0.00
Fresh Water, Hair 09	Original	0.02
	One week	0.00
	Two week	0.00
	Four week	0.00
	Eight week	0.00
Fresh Water, Hair 11	Original	0.04
	One week	0.00
	Two week	0.00
	Four week	0.00
	Eight week	0.00
Fresh Water, Hair 15	Original	0.00
	One week	0.00
	Two week	0.00
	Four week	0.00
	Eight week	0.00

Table 5.4 Concentration of AF in hair after immersion in sea water (ng/mg)

Sea Water	Hair 05	Hair 08	Hair 10	Hair 12	Hair 16	Hair 17
Original	16.52	2.08	1.69	0.17	12.38	57.69
One Day	4.68	0.81	0.66	0.17	3.93	8.57
Two Days	2.72	0.63	0.57	0.17	1.25	8.32
One Week	2.17	0.37	0.34	0.16	1.19	5.34
Two Weeks	1.09	0.32	0.22	0.10	0.90	3.18
Three Weeks	0.84	0.31	0.18	0.09	0.85	2.55
Four Weeks	0.79	0.22	0.16	0.09	0.62	2.18
Eight Weeks	0.65	0.13	0.10	0.02	0.49	1.40

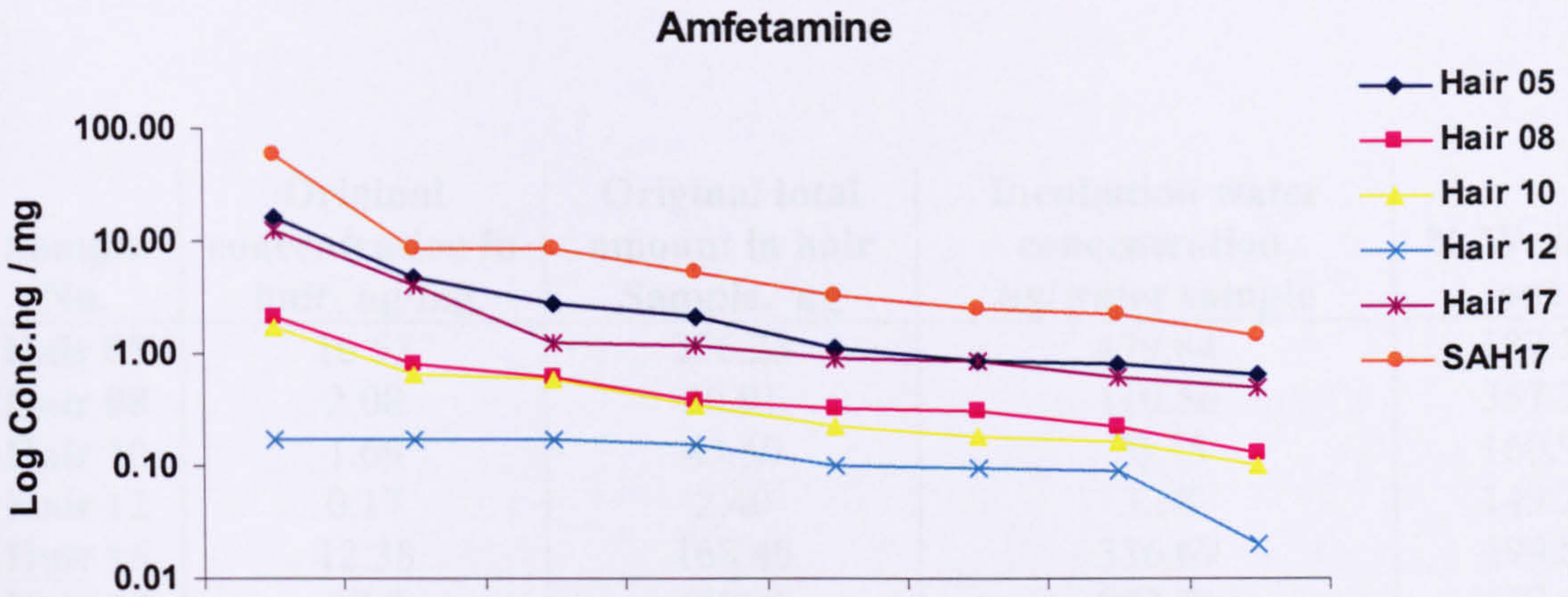


Figure 5.2 Effect of sea water immersion on AF concentration in hair

Table 5.5 Concentration of AF in hair after immersion in fresh water (ng/mg)

Fresh Water	Hair 04	Hair 09	Hair 11	Hair 15
Original	2.49	11.12	4.73	12.12
One Week	2.25	9.78	4.09	10.05
Two Weeks	1.90	9.46	3.95	8.89
Four Weeks	1.85	9.38	3.24	8.29
Eight Weeks	1.78	9.29	1.86	6.41

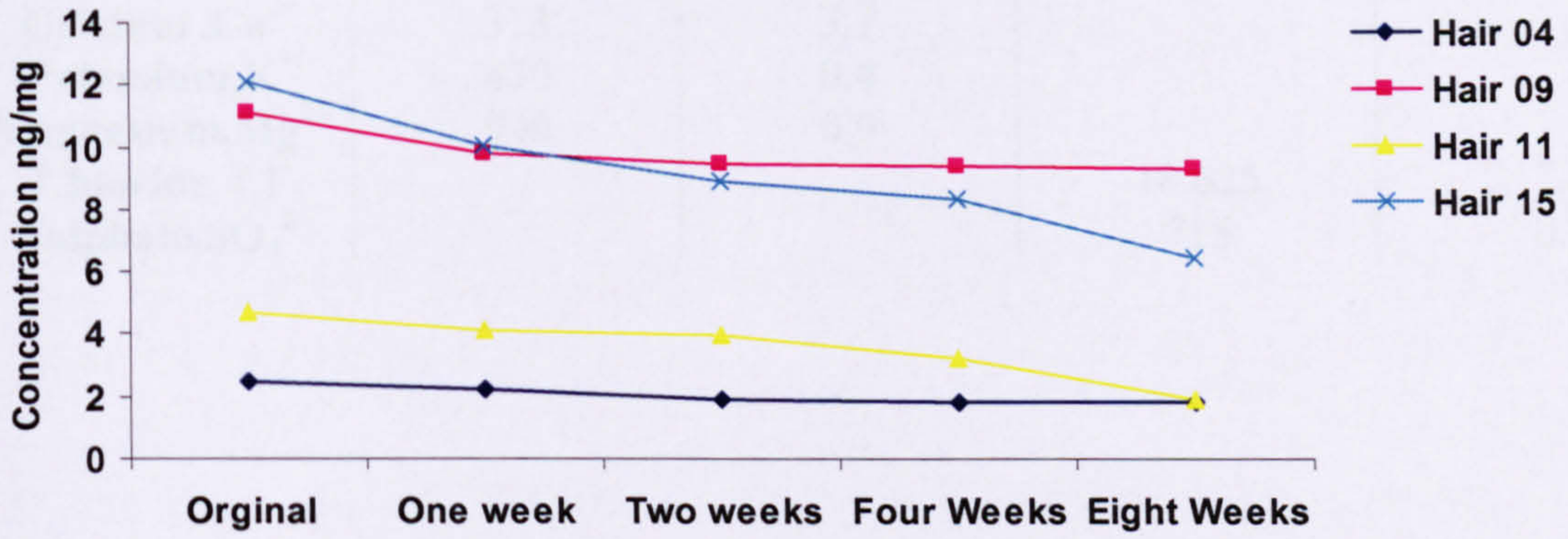


Figure 5.3 Effect of fresh water immersion on AF concentration in hair

Table 5.6 AF concentration in hair and sea water after eight weeks

Sample No.	Original concentration in hair, ng/mg	Original total amount in hair Sample, ng	Incubation water concentration, ng/water sample	% Washed out
Hair 05	16.52	256.23	479.84	187.3
Hair 08	2.08	30.91	110.56	357.7
Hair 10	1.69	43.60	70.15	160.9
Hair 12	0.17	2.40	3.58	149.2
Hair 16	12.38	168.49	336.69	199.8
Hair 17	57.7	777.8	992.76	127.6

Table 5.7 AF concentration in hair and fresh water after eight weeks

Sample No.	Original concentration in hair, ng/mg	Original total amount in hair Sample,ng	Incubation water concentration, ng/water sample	% Washed out
Hair 04	2.49	37.92	47.28	124.7
Hair 09	11.12	241.86	275.81	114.0
Hair 11	4.73	74.88	67.8	90.5
Hair 15	12.12	161.2	46.56	28.0

Table 5.8 Physical properties of water

	Cation Concentration (mg/L)		Anion Concentration (mg/L)	
Metal ions	**Sea Water**	**Fresh water**	**Sea Water**	**Fresh water**
Sodium, Na^+	9,800	6.2		
Calcium ,Ca^{2+}	313	3.7		
Potassium,K^+	450	0.4		
Magnesium,Mg^{2+}	940	0.9		
Chloride, Cl^-			18,625	7.8
Sulphate,SO_4^{2-}			715	0.8

6 Case studies

6.1 Introduction

Hair samples were collected from volunteers with a known history of Fenethylline (AF precursor) abuse and were subjects in rehabilitation programs at Alamal Medical Complex in Riyadh, Saudi Arabia. Urine samples previously received from these volunteers had been analysed by the laboratory at the Alamal Medical Complex in Riyadh and most of these were found to be positive for AF.

Hair samples were obtained by cutting from the area at the back of the head and as close to the scalp as possible at the posterior vertex region of the head. Once collected, these were stored at room temperature in aluminium foil and sealed in paper envelopes until use. 15 hair samples were obtained from the Alamal Medical Complex. One other case hair sample collected from a living individual within the UK was received by Forensic Medicine and Science. The hair samples were cut to give 0–3 cm lengths. 2 out of the 16 hair samples were cut to give a further three and six segments, each 3 cm long.

Case sample details were collected for each of the volunteers. These included sex and age of the individual, hair colour, information regarding drug use, date of admission to rehabilitation programme and the date of hair collection. These results are tabulated in Table 6.1.

Postmortem hair samples were obtained from the Department of Forensic Medicine and Science, University of Glasgow. These hair samples were pulled from the posterior vertex region of the scalp. Once collected, these were stored at room temperature in aluminium foil and sealed in paper envelopes until use. Hair case samples were selected depending on the results of the post-mortem blood sample received from the same case. If the blood was positive for AF or related compounds and a hair sample was available then these hair samples were selected. 6 case hair samples were obtained in these types of cases. The hair samples were cut to give 0–3 cm lengths.

For these cases, sex and age of the individual, hair colour, information regarding drug use and cause of death were recorded where available from police and pathology reports. These are shown in Table 6.2.

The validated method as described previously in section 4.3 was applied to the 22 case hair samples.

Table 6.1 Case Sample Details

Case No	Sex	Age	Colour	Drug Used	Regular Amount	Duration of Use	Segments Analysed(cm)	Admission Date	Collection Date
HairC01	Male	27	Black	Can Fen	5 cig/day 1 tab/day	> 9 years > 9 years	0-3	09/06/2004	22/07/2004
HairC02	Male	25	Black	Can Fen	Occasionally 3-4 tab/day	> 3 years > 3 years	0-3	08/07/2004	22/07/2004
HairC03	Male	38	Black	Can Fen	1 cig/day 1 tab/day	> 10 years > 5 years	0-3	02/07/2004	22/07/2004
Hair04	Male	24	Black	Can Fen	1 cig/day 1 tab/day	> 7 years > 8 years	0-3	19/07/2004	22/07/2004
Hair05	Male	32	Black	Fen	7 tab/day	> 7 years	0-3	04/07/2004	22/07/2004
Hair07	Male	27	Black	Can Fen	3-5cig/day unknown	> 6 years interval	0-3, 3-6, 6-9, 9-12, 12-15, 15-18	21/07/2004	22/07/2004
Hair08	Male	32	Black	Fen	2 tab/day	> 2 years	0-3	22/07/2004	24/07/2004
Hair09	Male	21	Black	Can Fen	1-2 cig/day 4-5 tab/day	> 3 years	0-3	21/07//2004	22/07/2004
Hair10	Male	20	Black	Can Fen	5 cig/day 4-5 tab/day	> 4 years	0-3	23/07/2004	24/07/2004
Hair11	Male	25	Black	Fen	1-5tab/day	> 7years	0-3	04/07/2004	24/07/2004
Hair12	Male	23	Black	Can Fen	unknown 4-5 tab/day	> 5 years > 7 years	0-3	28/06/2004	24/07/2004
Hair14	Male	34	Black	Fen	8-9 tab/day	> 8 years	0-3	25/07/2004	29/07/2004
Hair15	Male	35	Black	Can Fen	1 cig/day 2-3 tab/day	> 1 years > 3 years	0-3	25/07/2004	29/07/2004
Hair16	Male	40	Black	Fen	8 tab/day	> 15 years	0-3	20/07/2004	29/07/2004
Hair17	Male	26	Black	Can Fen	2 cig/day 3-4tab/day	> 10 years	0-3	24/07/2004	29/07/2004
Hair18	Female	9	Brown	AF	Unknown	Unknown	0-3, 3-6, 6-9	28/08/2003	03/09/2003

cig: cigarette, tab: tablet, Can: cannabinoids, Fen: fenethylline

Table 6.2 Postmortem Case Sample Details

Case No	Sex	Age	Colour	Amfetamines Used	Other Drug Used	Cause of Death	Regular Amount	Duration of Use	Segment Analysed(cm)
PM01	Male	28	Black	Unknown	Heroin, Alcohol, Methadone	Heroin, Alcohol Intoxication	Unknown	Unknown	0-3
PM02	Male	30	Black	Unknown		AF, Ecstasy Intoxication	Unknown	Unknown	0-3
PM03	Male	21	Brown	Ecstasy	Alcohol, Diazepam, Cannabinoids	Methadone Intoxication	Unknown	Unknown	0-3
PM04	Male	36	Brown	Unknown	Unknown	Heroin, Temazepam Intoxication	Unknown	Unknown	0-3
PM05	Male	40	Black	Ecstasy	Alcohol	Ecstasy, Amitriptyline Intoxication	Unknown	Unknown	0-3
PM06	Male	19	Black	Unknown	Alcohol Absinthe spirit	Alcohol Intoxication	Unknown	Unknown	0-3

6.2 Results and Discussion

Thirteen urine case sample results which were screened by the Alamal Medical Complex were positive for amfetamine. In each case, chronic amfetamine abuse was confirmed by the result of the hair analysis, where amfetamine was found in 0-3 cm of all hair samples and also in each segment of hair samples labelled as Hair07 and Hair18 in Table 6.1. All other amfetamines (MA, MDA, MDMA and MDEA) gave negative results. For all case samples, these results showed good qualitative correlation between self-reported use, urine result at admission and hair result for AF. These hair sample results were only positive for AF which is likely to have come from the use of the AF precursor, fenethylline, where 27% of the fenethylline dose is excreted as racemic amfetamine [79]. In this study, self-report showed good agreement with hair findings. The concentration of AF in hair ranged from 0.15 – 57.69 ng/mg. The results are summarized in Table 6.3. These values correspond with other studies. One study reported AF hair concentrations in the hair of 40 volunteer subjects ranging from 0.01-4.46 ng/mg. These individuals were on rehabilitation programs and were known drug users [65]. In another study the concentration of AF in hair ranged from 0.74-1.02 ng/mg in healthy young amfetamines users [87].

It was difficult in this study to try and directly correlate the dose with the amount of AF found in the hair because the dose of fenethylline in these cases was unknown. The self-reports of the volunteers showed that a variable number of fenethylline tablets were used. The exact dose of fenethylline in these cases was unknown i.e. tablets can be of varying doses. An attempt was made to find if there was any correlation between self-reported use and the amount of amfetamine found in hair. For this purpose, the dose of each tablet was assumed to be the same. The case hair samples were categorised into three different groups according to the number of fenethylline tablets used. These were low (1-2 tablets), medium (3-5 tablets) and high (> 5 tablets). Self-reports (amount of tablets) and concentration of AF in hair was compared. These results showed that there was overlap between the three levels. The lowest and highest levels found in hair fell into the medium category and not into the low and high categories respectively. This may be explained by by the varying dose in tablets and the inaccuracy of self-report data. These results are summarised in Table 6.5.

The effect of hair colour on the incorporation of AF into hair was eliminated in this study, because the majority of hair samples (15 out of 16) were the same colour (black).

This study was an initial attempt to provide information about the accuracy of self reports by analysing hair samples. All hair samples from individuals reporting to have abused Fenethylline were positive for AF showing that this was a reliable method for showing previous use and could be used to complement urine testing. Overall, this study indicated that there was a lack of correlation between self reported use and concentration of AF in hair. This will be partly due to varying dosages of the drug within tablets.

Hair18 (Table 6.3) was obtained from a different source. Case details were unknown for this particular sample. However, the hair sample was found to be positive along the length for AF indicating regular exposure.

Postmortem blood samples were analysed by Forensic Medicine and Science at University of Glasgow for amfetamines and related compounds. Chronic amfetamine or ecstasy abuse was confirmed by the results of the hair analysis. In many of these cases regular use was not known. The postmortem hair samples were positive for these drugs and in general the results showed that there was good qualitative correlation between blood and hair results. In some cases these were also supported by evidence in the police report. Postmortem hair samples that were positive for MDMA were also positive for its break down product, MDA. All of these samples were also positive for AF. This may be a result of taking AF tablets or alternatively as an impurity in ecstasy tablets. All other amfetamines (MA and MDEA) gave negative results for hair samples. The concentration of AF in hair ranged from 0.04 – 4.42 ng/mg. The levels in hair ranged from 0.13-5.39 ng/mg for MDMA and 0.04-0.78 ng/mg for MDA. The ratio of MDMA/MDA in the hair samples ranged from 3.25-11.10. In all of these cases the level of MDMA was higher than the level of MDA and it is likely the MDA is from the breakdown of MDMA.

Out of the 6 postmortem hair cases that were analysed, ecstasy was known to be used in 2 cases. Ecstasy use was not known for the other four cases, but MDMA and MDA were found in these blood and hair samples. The findings in the hair samples would tend to suggest regular use. The hair MDMA levels found in this study were similar to those that have been found in cases of known ecstasy users. It was reported that the concentration of MDMA from 15 regular users of MDMA and MA ranged from 1.20-12.6 ng/mg in 1cm hair. These volunteers had consumed 1 to 4 tablets with varying doses in the last month [92]. Another study found MDMA concentrations ranging from 0.117-6.44 ng/mg in 20 cases of known users [99].

The results are summarized in Table 6.4 and the chromatograms of the control sample for all five amfetamines and one of authentic sample for AF, MDA and MDMA are shown in

Figure 6.1, Figure 6.2, Figure 6.3, Figure 6.4, Figure 6.5, Figure 6.6, Figure 6.7 and Figure 6.8 respectively.

Table 6.3 Amfetamines concentration finding in hair and urine case Samples

Case Sample	Urine		Hair ng/mg
	AF	Cannabinoids	AF
HairC01	Neg	Pos	2.45
HairC02	Pos	Neg	1.64
HairC03	Pos	Pos	3.32
Hair04	Pos	Pos	2.49
Hair05	Pos	Neg	16.52
Hair07	Neg	Pos	0.65 (0-3 cm) 0.22 (3-6 cm) 0.39 (6-9 cm) 0.19 (9-12 cm) 0.17 (12-15cm) 0.15 (15-18cm)
Hair08	Pos	N/D	2.08
Hair09	Pos	Pos	11.12
Hair10	Pos	Pos	1.69
Hair11	Pos	N/D	4.73
Hair12	Pos	Pos	0.17
Hair14	Pos	N/D	41.25
Hair15	Pos	Pos	12.12
Hair16	Pos	N/D	12.37
Hair17	Pos	Pos	57.69
Hair18	Pos	N/D	0.53 (0-3 cm) 0.3 (3-6 cm) 0.46 (6-9 cm)

N/D: Not Done

Table 6.4 Amfetamines concentration finding in hair and blood in postmortem case Samples

	AF		MA		MDA		MDMA		MDEA	
Case Sample	Blood mg/L	Hair ng/mg	Blood mg/L	Hair ng/mg	Blood mg/L	Hair ng/mg	Blood mg/L	Hair ng/mg	Blood mg/L	Hair ng/mg
PM01	N/D	0.34	0.40	Neg	0.04	0.35	0.40	3.17	N/D	Neg
PM02	0.62	4.42	N/D	Neg	0.05	0.78	0.45	5.39	0.45	Neg
PM03	N/D	0.23	N/D	Neg	0.02	0.21	0.36	2.33	N/D	Neg
PM04	N/D	0.04	N/D	Neg	0.02	0.04	0.75	0.13	N/D	Neg
PM05	N/D	0.06	N/D	Neg	0.12	0.31	0.07	1.85	N/D	Neg
PM06	N/D	0.13	N/D	Neg	0.02	0.16	0.75	1.17	N/D	Neg

N/D: Not Done

Table 6.5 The concentration of AF and the number of tablets

No of tablet/day	Concentration of AF in hair (ng/mg)
Low (1-2)	2.08-3.32
Medium (3-5)	0.17-57.69
High >5	12.12-16.52

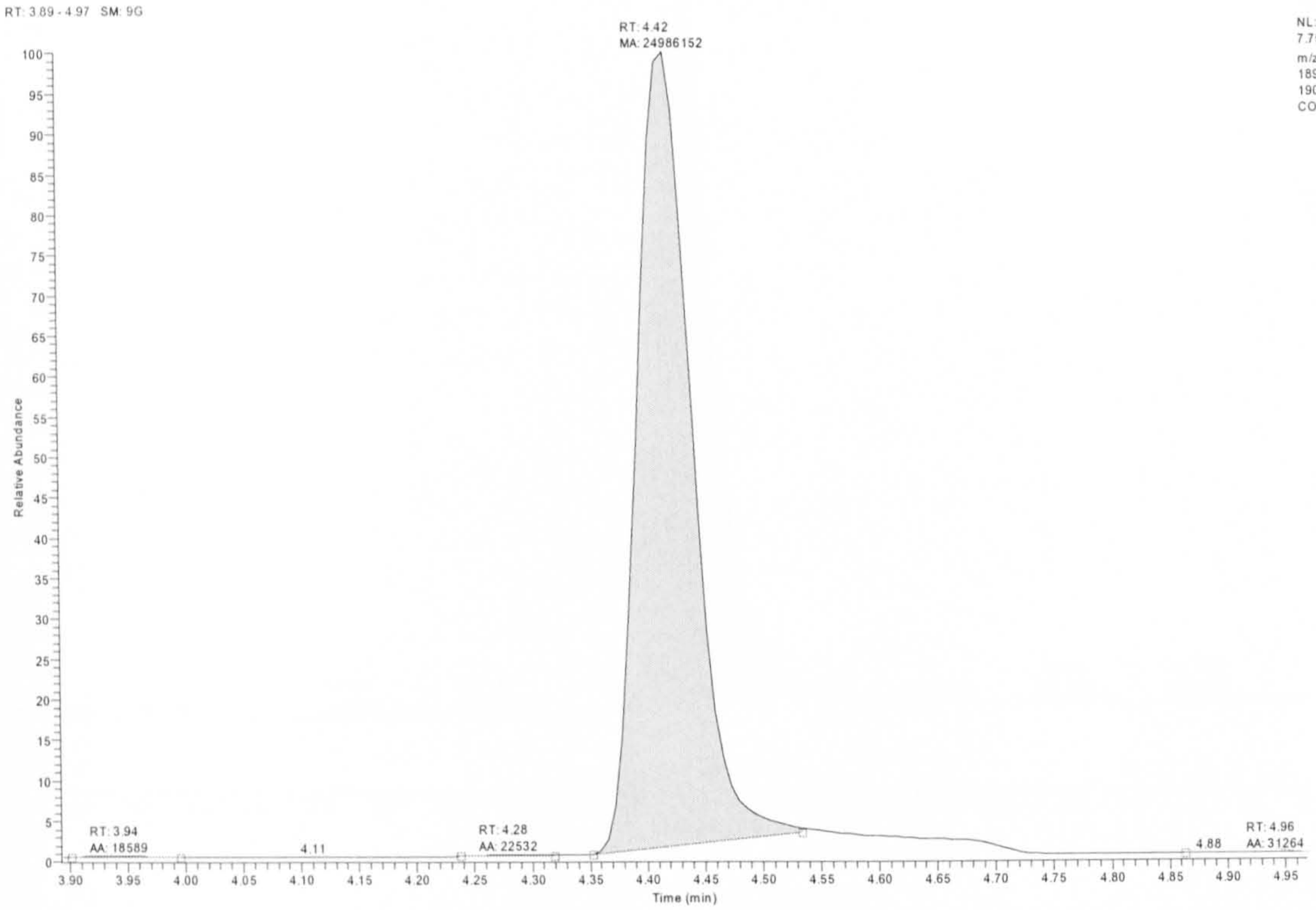


Figure 6.1 GC-MS-SIM Chromatogram of PFP derivative of AF control sample

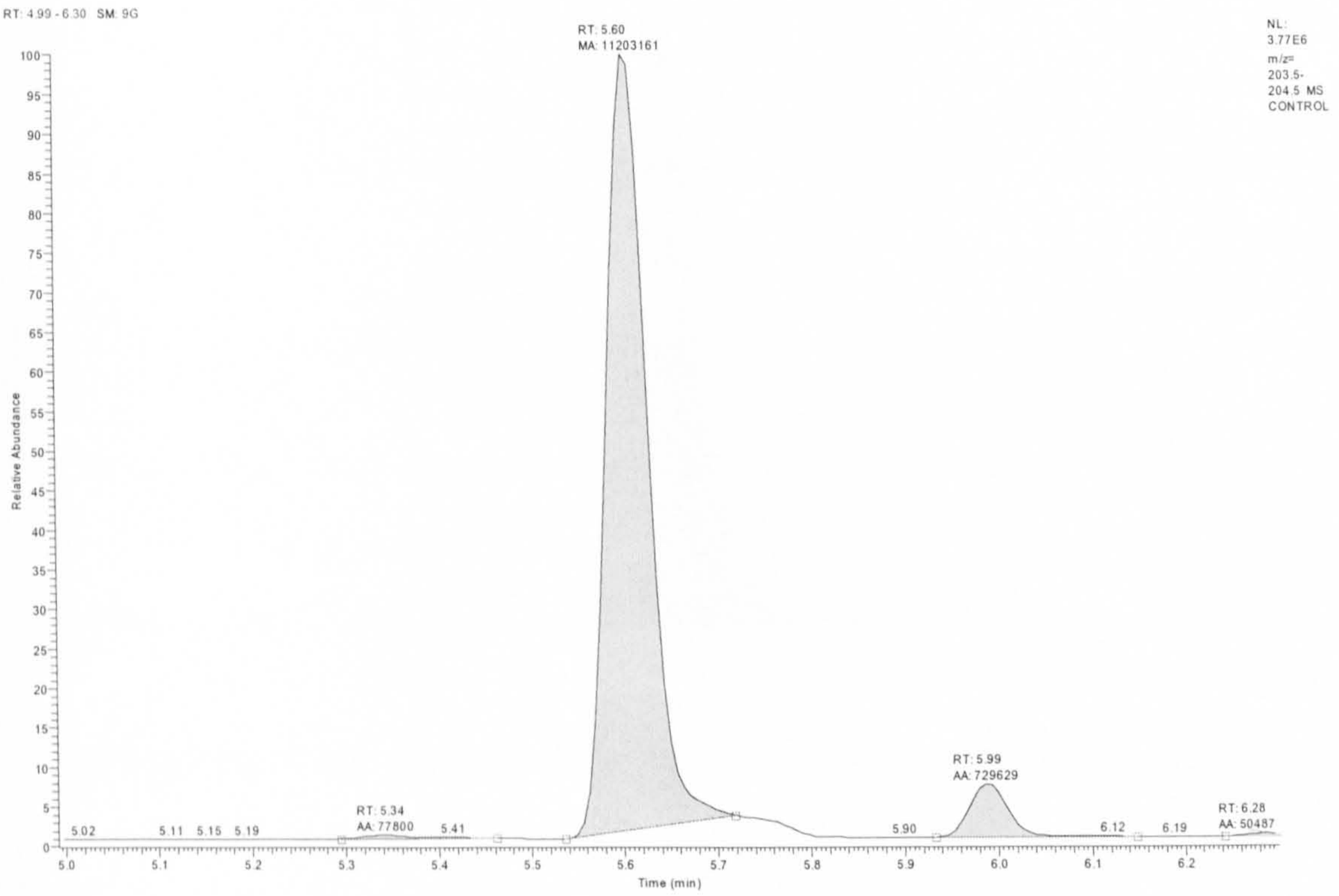


Figure 6.2 GC-MS-SIM Chromatogram of PFP derivative of MA control sample

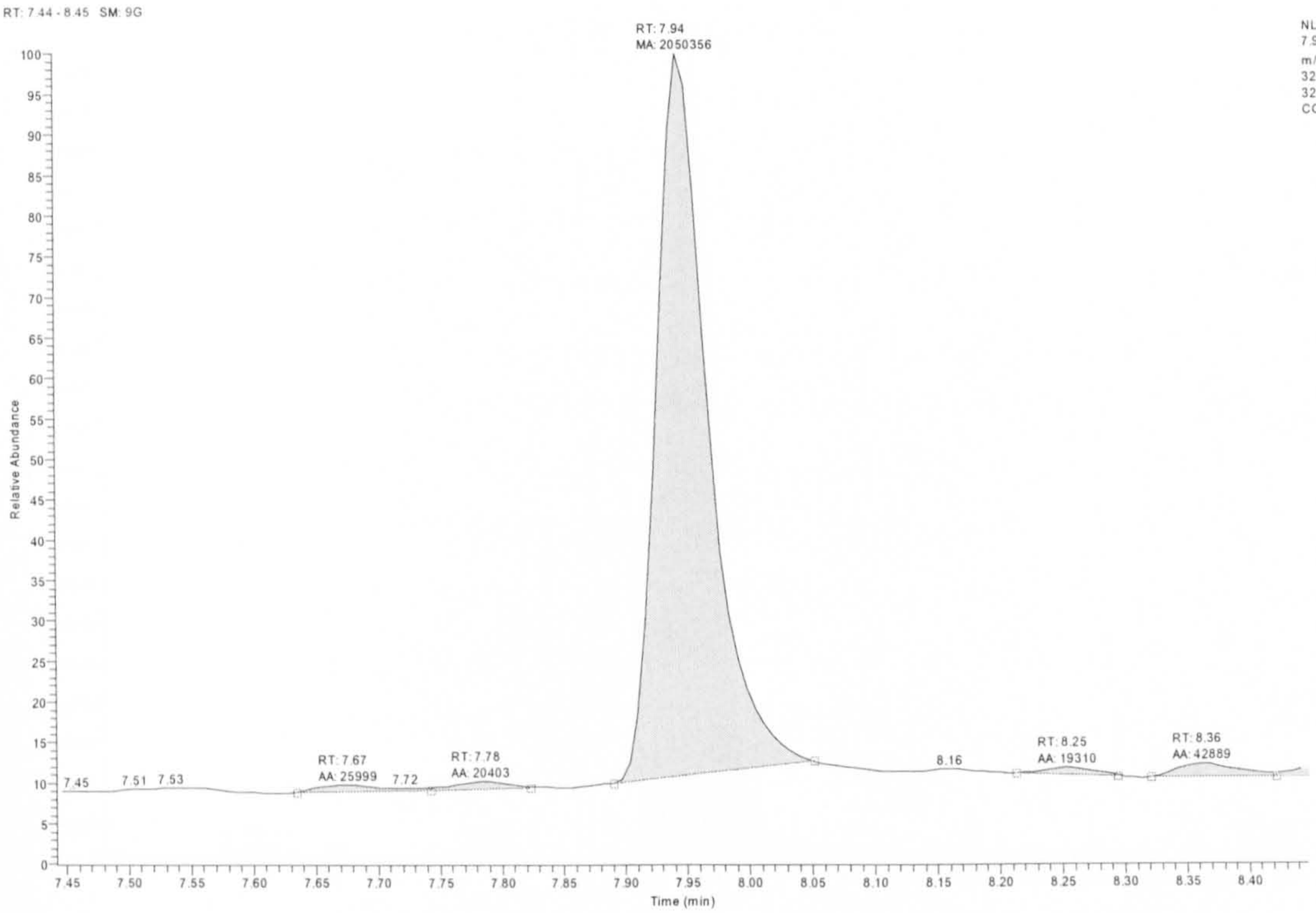


Figure 6.3 GC-MS-SIM Chromatogram of PFP derivative of MDA control sample

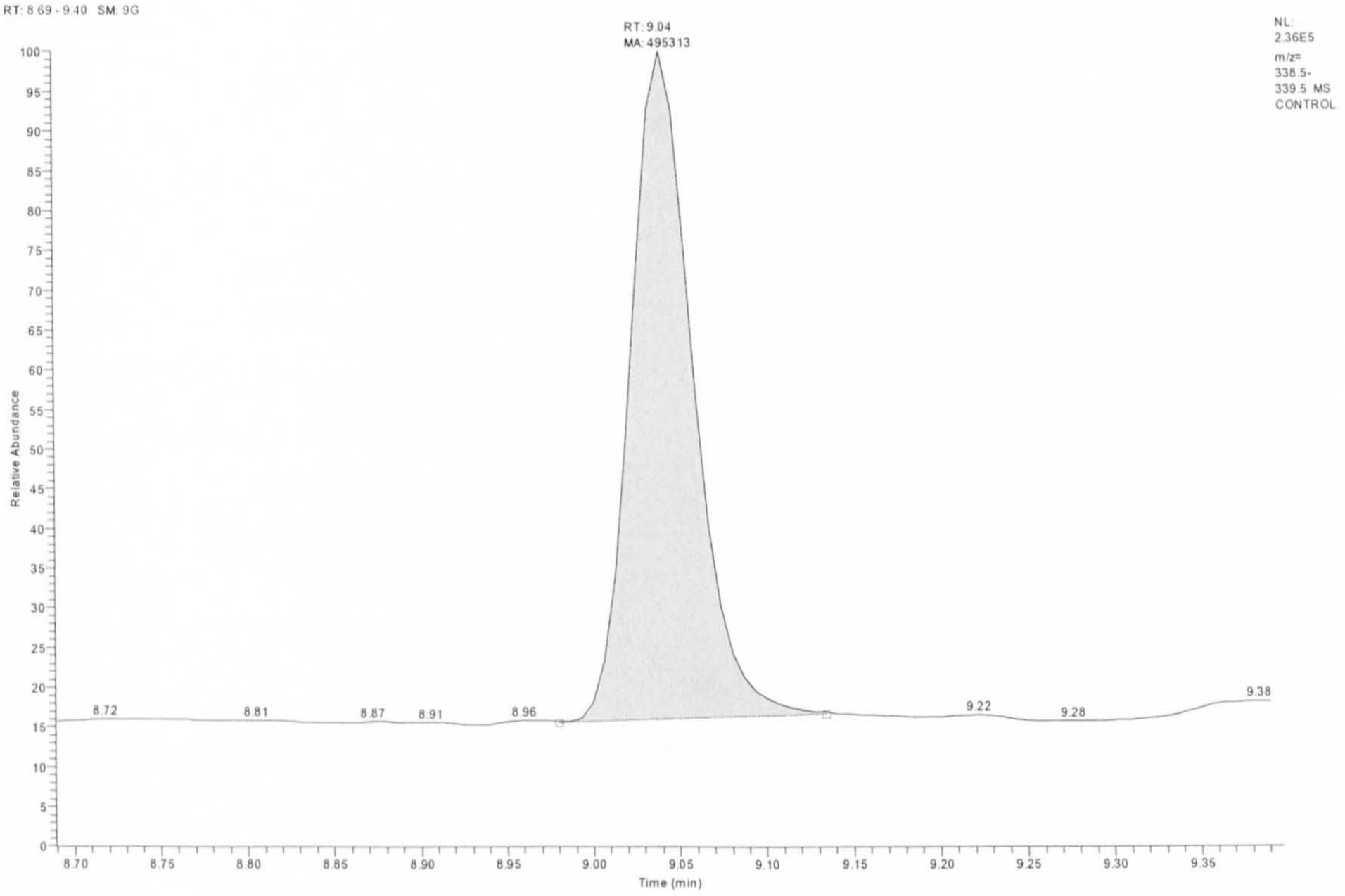


Figure 6.4 GC-MS-SIM Chromatogram of PFP derivative of MDMA control sample

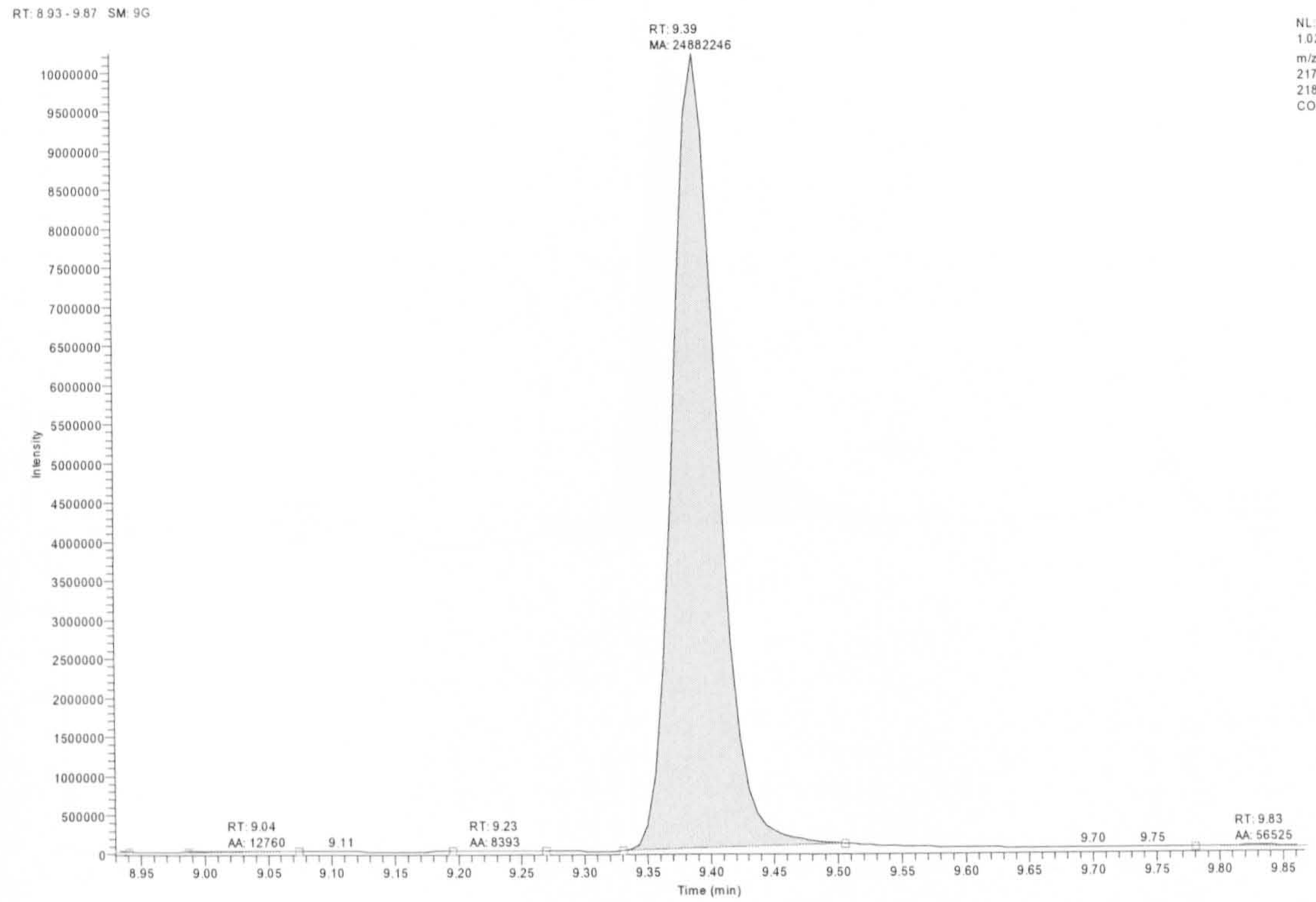


Figure 6.5 GC-MS-SIM Chromatogram of PFP derivative of MDEA control sample

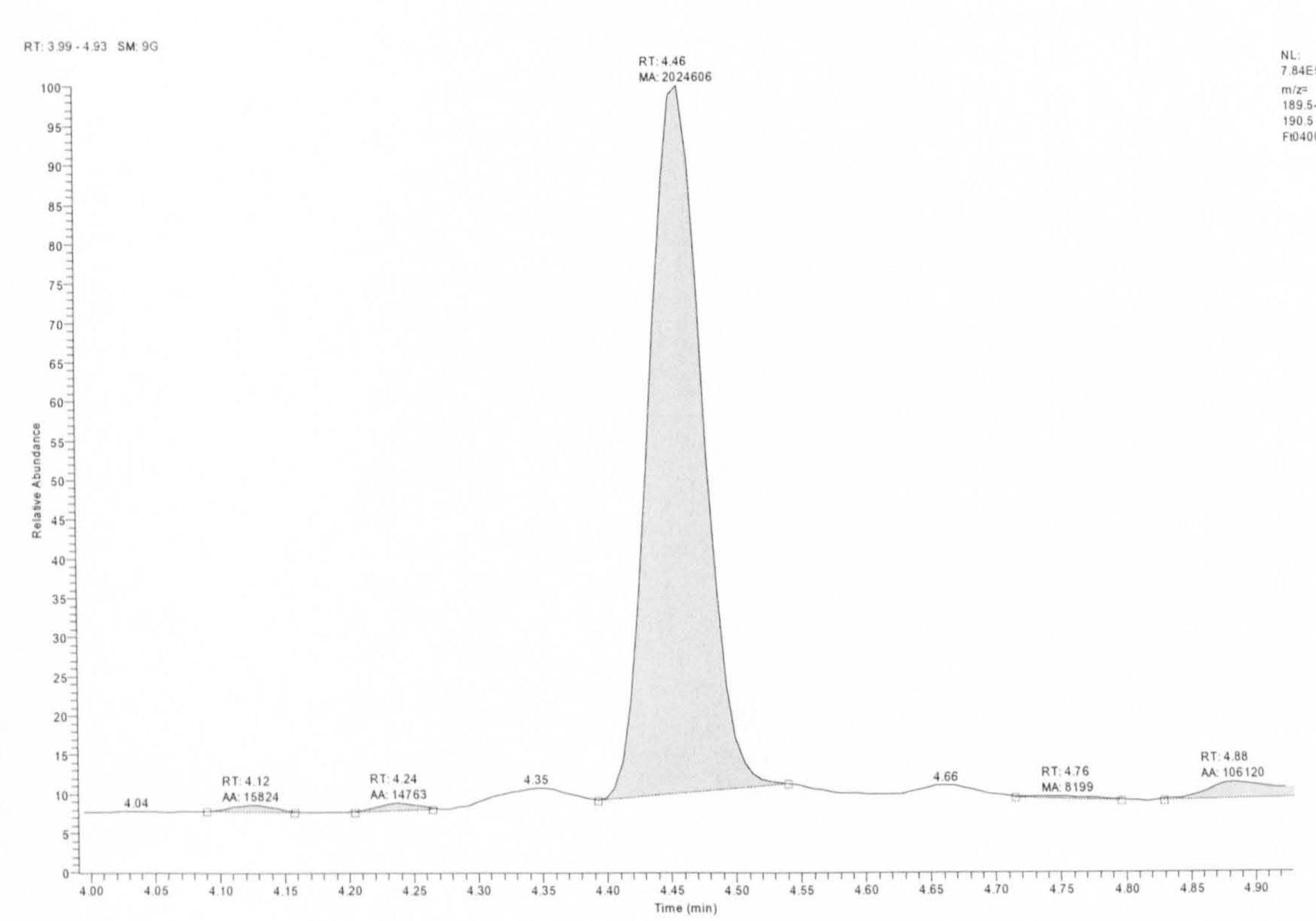


Figure 6.6 GC-MS-SIM Chromatogram of PFP derivative of AF anuthentic sample

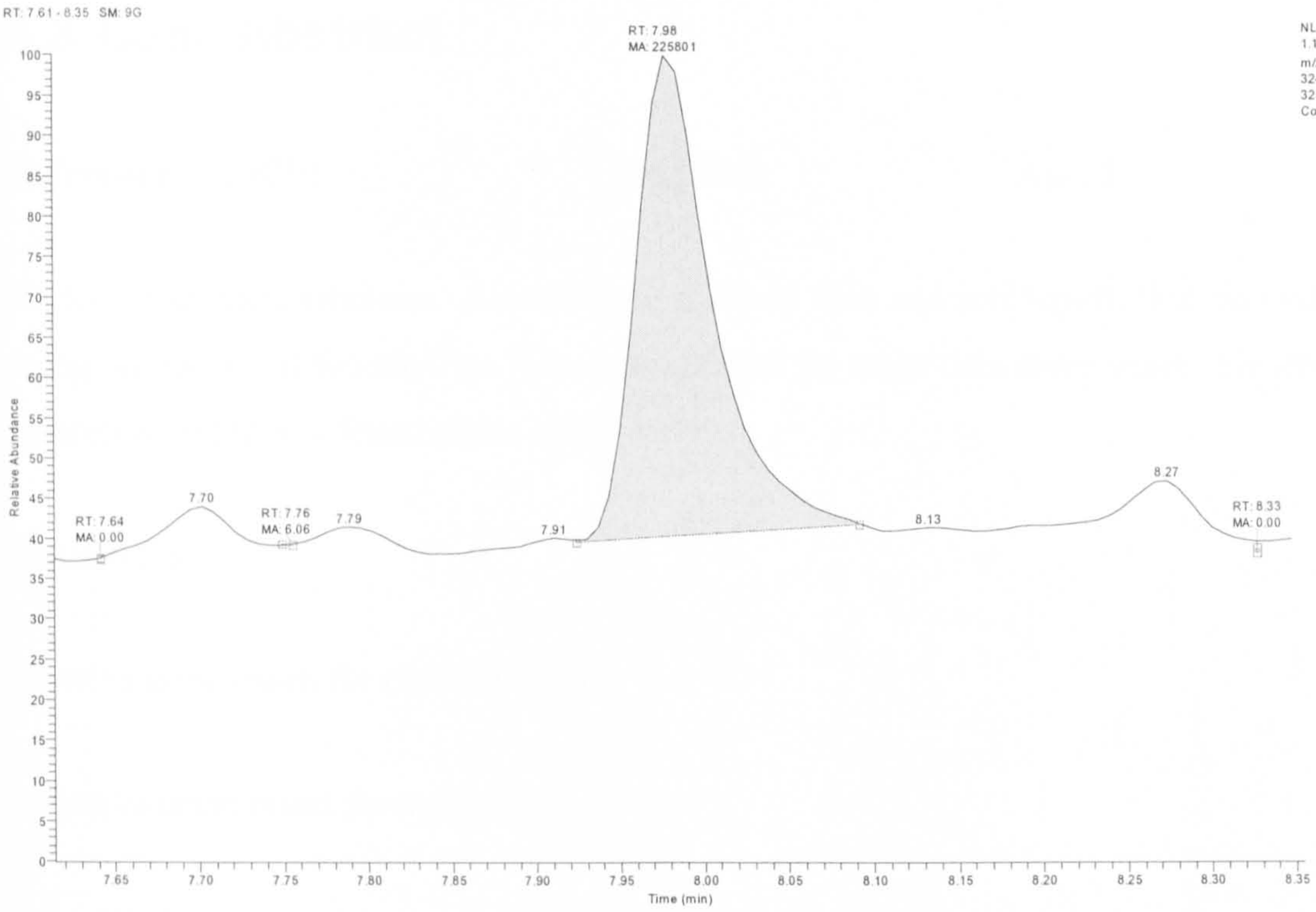


Figure 6.7 GC-MS-SIM Chromatogram of PFP derivative of MDA authentic sample

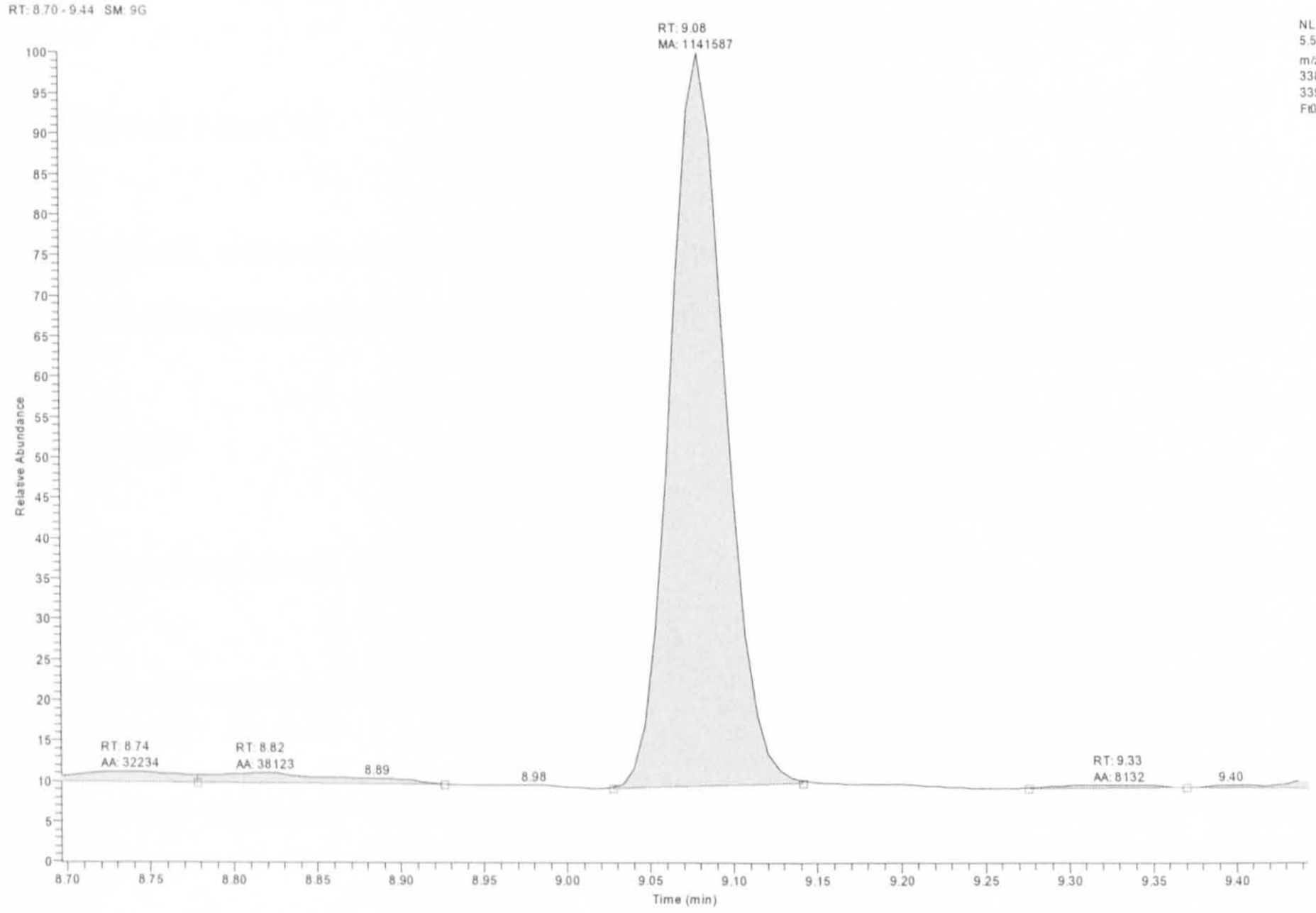


Figure 6.8 GC-MS-SIM Chromatogram of PFP derivative of MDMA authentic sample

6.3 Case Abstract

Reference: HairC01 **Sex**: Male **Age**: 27

Background circumstances: According to medical files and self report, this man had been using cannabis and fenethylline (captagon) tablets for more than three years. He smoked 5 cigarettes and took 1 fenethylline tablet daily.

Toxicology:

Positive urine result for cannabinoids.

Negative urine result for amfetamine.

0-3 cm hair segment:

2.45 nanograms of amfetamine per milligrams of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: HairC02 **Sex**: Male **Age**: 25

Background circumstances: According to his medical files and self report, this man had been smoking cannabis and taking 3-4 fenethylline tablets daily for more than three years

Toxicology:

Negative urine result for cannabinoids.

Positive urine result for amfetamine.

0-3 cm hair segment:

1.65 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: HairC03 **Sex:** Male **Age:** 38

Background circumstances: According to his medical files and self report, this man had been smoking 1 cannabis cigarette daily for more than ten years. He had also been taking 1 fenethylline tablet daily for more than five years.

Toxicology:

Positive urine result for cannabinoids.

Positive urine result for amfetamine.

0-3 cm hair segment:

3.32 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA)analyses gave NEGATIVE results.

Reference: Hair04 **Sex:** Male **Age:** 24

Background circumstances: According to his medical report and self report, this man had been smoking cannabis daily for more than seven years and taken fenethylline tablets for more than eight years. He also used alcohol occasionally.

Toxicology:

Positive urine result for cannabinoids.

Positive urine result for amfetamine.

0-3 cm hair segment:

2.49 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair05 **Sex:** Male **Age:** 32

Background circumstances: According to his medical files and self report, this man had been using 7 tablets of fenethylline daily for more than two years.

Toxicology:

Negative urine result for cannabinoids.

Positive urine result for amfetamine.

0-3 cm hair segment:

16.52 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair07 **Sex:** Male **Age:** 27

Background circumstances: According to his medical files, emergency room assessment and self report this man had been using cannabis for more than six years and had taken fenethylline tablets and used alcohol at intervals. He had smoked 3-5 cigarettes daily. A long strand of hair was collected and cut into six segments (0-3, 3-6, 6-9, 9-12, 12 -15 and 15-18 cm).

Toxicology:

Positive urine result for cannabinoids.

Negative urine result for amfetamine.

0-18 cm hair segment:

0.65 nanograms of amfetamine per milligram of hair in 0-3 cm segment.

0.22 nanograms of amfetamine per milligram of hair in 3-6 cm segment.

0.39 nanograms of amfetamine per milligram of hair for 6-9 cm segment.

0.19 nanograms of amfetamine per milligram of hair for 9-12 cm segment.

0.17 nanograms of amfetamine per milligram of hair for 12-15 cm segment.

0.15 nanograms of amfetamine per milligram of hair for 15-18 cm segment.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair08 **Sex**: Male **Age**: 32

Background circumstances: According to his medical files and self report, this man had been using 2 tablets of fenethylline daily for more than two years.

Toxicology:

Positive urine result for amfetamine.

0-3cm hair segment:

2.08 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair09 **Sex**: Male **Age**: 21

Background circumstances: According to his medical files and self report, this man had been using cannabis more than three years and had taken fenethylline tablets for more than three years. He smoked 1-2 cannabis cigarettes daily and took 4-5 fenethylline tablets daily.

Toxicology:

Positive urine result for cannabinoids.

Positive urine result for amfetamine.

0-3cm hair segment:

11.12 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair10 **Sex:** Male **Age:** 20

Background circumstances: According to his medical files and self report, this man had been using cannabis for more than four years and had taken fenethylline tablets for more than four years. He had smoked 5 cigarettes daily and had taken 4-5 fenethylline tablets daily.

Toxicology:

Positive urine result for cannabinoids.

Positive urine result for amfetamine.

0-3cm hair segment:

1.69 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair11 **Sex:** Male **Age:** 25

Background circumstances: According to his medical files and self report, this man had been using 1-5 tablets of fenethylline daily for more than seven years.

Toxicology:

Positive urine result for amfetamine.

0-3cm hair segment:

4.73 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair12 **Sex:** Male **Age:** 23

Background circumstances: According to his medical files and self report, this man had been using cannabis for more than five years and fenethylline tablets for more than seven years. He had smoked daily and had taken 9 fenethylline tablets daily and used alcohol occasionally.

Toxicology:

Positive urine result for cannabinoids.

Positive urine result for amfetamine.

0-3cm hair segment:

0.17 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair14 **Sex:** Male **Age:** 34

Background circumstances: According to his medical files and self report this man had been using 8-9 tablets of fenethylline daily for more than eight years.

Toxicology:

Positive urine result for amfetamine.

0-3cm hair segment:

41.25 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair15 **Sex:** Male **Age:** 35

Background circumstances: According to his medical files and self report, this man had been using cannabis more than one year and had taken fenethylline tablets for more than three years. 1 cigarette was smoked daily and he had taken 2-3 fenethylline tablets daily and used alcohol occasionally.

Toxicology:

Positive urine result for cannabinoids.

Positive urine result for amfetamine.

0-3cm hair segment:

12.12 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair16 **Sex:** Male **Age:** 40

Background circumstances: According to his medical report, this man had been using eight tablets of fenethylline daily for more than fifteen years.

Toxicology:

Positive urine result for amfetamine.

0-3cm hair segment:

12.37 nanograms of Amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair17 **Sex:** Male **Age:** 26

Background circumstances: According to his medical files and self report, this man had been using cannabis for more than ten year and he had taken fenethylline tablets for more than ten years. 2 cigarettes were smoked daily, 3-4 fenethylline tablets were taken daily and alcohol was used at intervals.

Toxicology:

Positive urine result for cannabinoids.

Positive urine result for amfetamine.

0-3cm hair segment:

57.69 nanograms of amfetamine per milligram of hair.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results.

Reference: Hair18 **Sex:** F **Age:** 9

Background circumstances: This child had been given amfetamine by her mother. A neighbour called the police and they attended the house to find that the child was unwell and in need of medical treatment. At the hospital, she was treated accordingly and her urine was found to be positive for amfetamine. Hair samples were taken shortly afterwards to establish if there was any evidence of previous amfetamine use.

Toxicology:

Positive urine result for amfetamine.

0-9 cm hair segment:

0.53 nanograms of amfetamine per milligram of hair in 0-3 cm segment.

0.3 nanograms of amfetamine per milligram of hair in 3-6 cm segment.

0.46 nanograms of amfetamine per milligram of hair in 6-9 cm segment.

All other amfetamines (MA, MDA, MDMA and MDEA) analyses gave NEGATIVE results for all segments.

Reference: PM01 **Sex**: Male **Age**: 28

Background circumstances: This young man had a history of alcohol and heroin abuse and had at times been using methadone. He was on a number of medications including antidepressants. He returned to his house from a party and appeared to be intoxicated. He was left lying on a couch in the living room and was later found to be unresponsive. He was taken to hospital and was found to be dead on arrival.

Cause of Death: Heroin and alcohol intoxication.

Toxicology:

0.4 milligrams of methamfetamine per litre of blood.

0.04 milligrams of methylenedioxyamfetamine per litre of blood.

0.4 milligrams of methylendioxymethylamfetamine per litre of blood.

0.44 milligrams of citalopram per litre of blood.

0.02 milligrams of desmethyldiazepam per litre of blood .

0.06 milligrams of codeine per litre of blood.

0.1 milligrams of morphine per litre of blood.

16.71 milligrams of paracetamol per litre of blood.

51 milligrams of alcohol per 100 millilitres of blood.

215 milligrams of alcohol per 100 millilitres of urine.

0-3 cm hair segment:

0.34 nanograms of amfetamine per milligram of hair.

0.35 nanograms of methylendioxyamfetamine per milligram of hair.

3.17 nanograms of methylendioxymethylamfetamine per milligram of hair.

All other amfetamines (MA and MDEA) analyses gave NEGATIVE results.

Reference: PM02 **Sex:** Male **Age:** 30

Background circumstances: This young man was known to abuse drugs but little was known about his medical history. He was found dead at his home seated on a chair slouched backwards, and beside him was a broken piece of mirror with white powder on it.

Cause of Death: Amfetamine and MDMA (ecstasy) intoxication.

Toxicology:

0.62 nanograms of amfetamine per litre of blood.

0.053 milligrams of methylendioxyamfetamine per litre of blood.

0.45 milligrams of methylendioxymethylamfetamine per litre of blood.

0.02 milligrams of diazepam per litre of blood.

0.17 milligrams of benzoylecgonine per litre of blood.

0.19 milligrams of methadone per litre of blood.

0.020 milligrams of dihydrocodeine per litre of blood.

0-3 cm hair segment:

4.42 nanograms of amfetamine per milligram of hair.

0.78 nanograms of methylendioxyamfetamine per milligram of hair.

5.39 nanograms of methylendioxymethylamfetamine per milligram of hair.

All other amfetamines (MA and MDEA) analyses gave NEGATIVE results.

Reference: PM03 **Sex:** M **Age:** 21

Background circumstances: This young man had a history of alcohol abuse and took drugs, including ecstasy and diazepam. He was drinking heavily and smoking cannabis and subsequently went to bed. He was found lying on his back in bed, apparently dead. An ambulance was called but he was confirmed to be dead.

Cause of Death: Methadone intoxication.

Toxicology:

0.02 milligrams of methylendioxyamfetamine per litre of blood.

0.36 milligrams of methylendioxymethylamfetamine per litre of blood.

0.08 milligrams of desmethyldiazepam per litre of blood.

0.04 milligrams of diazepam per litre of blood.

0.19 milligrams of methadone per litre of blood.

0.003 milligrams of dihydrocodeine per litre of blood.

0.017 milligrams of morphine per litre of blood.

0-3 cm hair segment:

0.23 nanograms of amfetamine per milligram of hair.

0.21 nanograms of methylendioxyamfetamine per milligram of hair.

2.33 nanograms of methylendioxymethylamfetamine per milligram of hair.

All other amfetamines (MA and MDEA) analyses gave NEGATIVE results.

Reference: PM04 **Sex:** M **Age:** 36

Background circumstances: This man had a long standing history of drug abuse and was positive for hepatitis B. He was found lying on the floor, dead. An ambulance attended at the scene but there were no vital signs. An uncapped syringe was found lying close to him.

Cause of Death: Heroin and temazepam intoxication.

Toxicology:

0.022 milligrams of methylendioxyamfetamine per litre of blood.

0.075 milligrams of methylendioxymethylamfetamine per litre of blood.

0.39 milligrams of oxazepam per litre of blood.

12.55 milligrams of temazepam per litre of blood.

0.021 milligrams of 6-monoacetylmorphine per litre of blood.

0.164 milligrams of codeine per litre of blood.

0.984 milligrams of morphine per litre of blood.

0-3 cm hair segment:

0.04 nanograms of amfetamine per milligram of hair.

0.04 nanograms of methylendioxyamfetamine per milligram of hair

0.13 nanograms of methylendioxymethylamfetamine per milligram of hair.

All other amfetamines (MA and MDEA) analyses gave NEGATIVE results.

Reference: PM05 **Sex:** M **Age:** 40

Background circumstances: This man returned home apparently drunk and continued drinking at home. The following morning he was found to be unresponsive and not breathing. Subsequent searching of his clothing revealed a single white tablet in one of the pockets, possibly thought to be ecstasy.

Cause of Death: MDMA (ecstasy) and amitriptyline intoxication.

Toxicology:

0.12 milligrams of methylendioxyamfetamine per litre of blood.

0.075 milligrams of methylendioxymethylamfetamine per litre of blood.

0.04 milligrams of desmethyldiazepam per litre of blood.

0.04 milligrams of diazepam per litre of blood.

0.36 milligrams of dihydrocodeine per litre of blood.

24.74 milligrams of paracetamol per litre of blood.

3.12 milligrams of amitriptyline per litre of blood.

1.99 milligrams of nortriptyline per litre of blood.

153 milligrams of alcohol per 100 millilitres of blood.

260 milligrams of alcohol per 100 millilitres of urine.

0-3 cm hair segment:

0.06 nanograms of amfetamine per milligram of hair.

0.31 nanograms of methylendioxyamfetamine per milligram of hair

1.85 nanograms of methylendioxymethylamfetamine per milligram of hair.

All other amfetamines (MA and MDEA) analyses gave NEGATIVE results.

Reference: PM06 **Sex**: M **Age**: 19

Background circumstances: This man had consumed a large amount of alcohol and was seen to consume a quantity of absinthe spirit (70% abv) and he appeared to become extremely drowsy and fell asleep. He was placed in a bedroom. Periodic checks were made on him but about an hour and a half after being put on the bed he appeared to turn blue. Despite resuscitation attempts he was pronounced dead.

Cause of Death: Acute alcohol intoxication.

Toxicology:

0.02 milligrams of methylendioxyamfetamine per litre of blood.

0.75 milligrams of methylendioxymethylamfetamine per litre of blood.

0.046 milligrams of lignocaine per litre of blood.

350 milligrams of alcohol per 100 millilitres of blood.

422 milligrams of alcohol per 100 millilitres of urine.

0-3 cm hair segment :

0.13 nanograms of amfetamine per milligram of hair.

0.16 nanograms of methylendioxyamfetamine per milligram of hair

1.17 nanograms of methylendioxymethylamfetamine per milligram of hair.

All other amfetamines (MA and MDEA) analyses gave NEGATIVE results.

7 Conclusion

Hair analysis has been shown to be effective in determining chronic use of drugs. This biological sample is advantageous in cases where it is suspected that urine specimens have been adulterated. Hair is difficult to adulterate, but if this is suspected then another sample can be requested easily. Amfetamines and cannabinoids are the two most abused substances in Saudi Arabia. For this reason a method to analyse both substances in hair simultaneously was investigated. Initial experiments showed that hair samples spiked with amfetamines gave good linearities using four different pre-treatment methods (alkaline pre-treatment using 1M NaOH, β-Glucuronidase using *helix pomatia*, methanol and acid pre-treatment using 0.1M HCl) followed by SPE and GC-MS analysis. However, the recoveries of cannabinoids using these methods were poor as shown by the inability to detect low concentrations of standards. The recovery of these drugs using the SPE method alone without pre-treatment was investigated and was found to be acceptable for these substances. The alkaline pre-treatment method was then modified using different pH (5, 6, 7 and 8) of the phosphate buffer and four different eluents, acetone / chloroform (1:1 v/v), hexane / EtOAc (9:1 v/v), hexane / EtOAc (8:2 v/v) and methanol to improve the cannabinoids (Δ^9 -THC and Δ^9 -THC-COOH) recovery. At the same time the amfetamines recoveries were monitored to ensure that their recoveries did not significantly decrease. Unfortunately the modified method demonstrated no significant improvement of cannabinoids recovery. A comparison study was carried out for the four pre-treatment methods to investigate which method gave the best recovery of amfetamine from hair using a hair case sample known to be positive for AF. This work showed that all four pre-treatment methods were effective in the extraction of AF from hair. However, it was shown that the best recovery for AF extraction was obtained using the β- glucuronidase pre-treatment method. The method was successfully validated for all amfetamine compounds.

To assess the validity of analysing hair samples for the presence of AF in victims of drowning, the change in concentration of AF in hair submerged in sea water and fresh water over a period of time was investigated. Ten amfetamine positive hair samples were submerged in fresh and sea water for different periods of time. Six of these specimens were separated into 8 portions each. One of these portions was stored as an original specimen and 7 portions were immersed in 5 ml of sea water for 1, 2 days, 1, 2, 3, 4 or 8 weeks at 5 °C. The other four specimens were separated into 5 portions, one of these portions was stored as an original specimen and 4 were stored in 5 ml of fresh water for 1, 2, 4 or 8

weeks at 5 °C. The drug concentrations in the samples were monitored over this period. Hair samples were analysed using the validated method. The results showed a significant decrease of AF in hair with the time submerged in sea water. Fresh water had a much less significant effect over the study period. The decrease in hair AF concentration after one day in sea water ranged from 0.1-85% and after eight weeks ranged from 88-97%. In fresh water, after one week the concentration was found to decrease by 9-17% and after eight weeks by 16-60%.

The incubation water used to submerge the samples after eight weeks was found to be positive for AF. The sea water contained a higher concentration of metal ions than the fresh water. The high concentration of metal ions in sea water may explain the increased rate of removal of AF from hair when compared to fresh water.

Careful interpretation is required for hair samples obtained from victims of drowning. Sea water has the potential to cause the complete loss of drug from hair with increased time, while, fresh water may cause partial or possible complete loss with increased time.

A total of 22 living and post-mortem cases samples were analysed by the validated method. Control hair samples were run simultaneously with case samples and their results were found to be within an acceptable range. This method is appropriate for the identification of amfetamines drugs in hair. Chronic amfetamine or ecstasy abuse was confirmed by the results of the hair analysis. For all case samples, these results showed good qualitative correlation between self-reported use, urine or blood results and hair results for amfetamines.

7.1 Further Work

In accordance with medical files and self reports of AF abusers involved in rehabilitation programs at Alamal Medical Complex in Riyadh, Saudi Arabia, positive AF results were attributed to the illegal precursor of AF called fenethylline (captagon). The development of a method capable of detecting the parent drug, fenethylline and its metabolites AF simultaneously would be beneficial in order to discriminate between the source of AF. The extent of fenethylline use could then be recognized. Also, since theophylline is fenethylline metabolite, its identification may be of some use.

Further work could be carried out on the AF stability study to include AF related compounds using different storage temperatures (refrigerator and freezer), different incubation times, different ionic strengths and different incubation conditions (dark, light, UV, pH).

The proposed method requires further investigations to improve the cannabinoids recoveries to a satisfactory degree and to be able to include them in the method. More sensitive instrumentation would be beneficial to improve the cannabinoids detection in hair.

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9 Appendix One: Presentation in Support of this Thesis

Abstract Only

- Fahad N. Bin-Eisa, Fiona M. Wylie and John S. Oliver. An investigation of the stability of amfetamine in hair samples submerged in a fresh and sea water environment. Proceeding of the TIAFT 2005 Meeting, Seoul, Korea, (2005).

An investigation of the stability of amphetamine in hair samples submerged in a fresh and sea water environment.

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Abstract

To assess the validity of analysing hair samples for the presence of amphetamine in victims of drowning, a method was first established to identify and measure the concentration of this drug in hair specimens. Amphetamine was extracted from hair using enzyme treatment with β-Glucuronidase (helix, pomatia) at 45 °C for 2 hours followed by solid phase extraction using Bond Elut Certify, mixed-mode columns. The samples were analysed by gas chromatography-mass spectrometry. An. HP-5 capillary column, 30 m x 0.32 mm x 0.25 μm film thickness was used and the temperature was programmed from 100 to 300 °C at a rate of 12°C / minute and held at 300 °C for 5 minutes. The method was validated and found to be linear over the concentration range 5-200 nanograms per 30 milligrams of hair with a correlation co-efficient of 0.999. The limits of detection and quantitation were 2.7 nanograms per 30 milligrams of hair and 4.3 nanograms per 30 milligrams of hair respectively. Intra-day reproducibility had coefficients of variation of less than 10 % at 3 different analyte concentrations (10, 50 and 200 nanograms per 30 milligrams of hair). Inter-day reproducibility coefficient of variation was less than 15% and the recovery of the method was 71 %.

The effect of salt water and fresh water on the concentration of amphetamine in hair specimens was investigated by splitting known amphetamine positive specimens. These were stored in these types of water at 5 °C for specific time intervals ranging from one day to eight weeks. The initial concentrations of the hair samples investigated ranged from 0.14 to 57.70 nanograms per milligram of hair The results showed a significant decrease of amphetamine in hair with incubation time in salt water. The decrease in the different samples after eight weeks of incubation in salt water ranged from 60-95%. Fresh water had a much less significant effect over the study period.

Keywords: amphetamine, hair, stability